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Maurizio Lambardi  
Elif Aylin Ozudogru  
Shri Mohan Jain *Editors*

Protocols  
for Micropropagation  
of Selected  
Economically-Important  
Horticultural Plants

 Humana Press

# METHODS IN MOLECULAR BIOLOGY™

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**John M. Walker**  
School of Life Sciences  
University of Hertfordshire  
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# **Protocols for Micropropagation of Selected Economically-Important Horticultural Plants**

Edited by

**Maurizio Lambardi**

*IVALSA-Istituto per la Valorizzazione del Legno e delle Specie Arboree,  
National Research Council (CNR), Sesto Fiorentino, Florence, Italy*

**Elif Aylin Ozudogru**

*IVALSA-Istituto per la Valorizzazione del Legno e delle Specie Arboree,  
National Research Council (CNR), Sesto Fiorentino, Florence, Italy*

**Shri Mohan Jain**

*Department of Agricultural Sciences, University of Helsinki, Helsinki, Finland*

*Editors*

Maurizio Lambardi  
IVALSA-Istituto per la Valorizzazione  
del Legno e delle Specie Arboree  
National Research Council (CNR)  
Sesto Fiorentino, Florence, Italy

Elif Aylin Ozudogru  
IVALSA-Istituto per la Valorizzazione  
del Legno e delle Specie Arboree  
National Research Council (CNR)  
Sesto Fiorentino, Florence, Italy

Shri Mohan Jain  
Department of Agricultural Sciences  
University of Helsinki  
Helsinki, Finland

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## Foreword

Tissue culture and micropropagation are exciting areas of biotechnology due to the combination of high scientific complexity with an enormous horticultural impact. Horticulture makes extensive use of tissue culture to maintain or grow plant cells and organs in vitro for a wide range of applications in plant propagation, such as breeding, plant pathology, or germplasm conservation. Therefore, a new book on micropropagation of economically important horticultural plants is welcome and needed, since it will contribute to the scientific progress of a high relevant area of horticultural technology.

Current micropropagation procedures used by the industry are the result of basic and applied research in biology, plant physiology, and genetics. Technical progress in micropropagation is intertwined with many important discoveries in plant science. We may say that modern tissue culture is the result of pyramiding key scientific discoveries during more than a century of research.

This book is focused on protocols, but it smartly combines the scientific principles with the state of the art in tissue culture techniques presented by experienced and prestigious authors. The editors are recognized scientists and all chapters were peer-reviewed, which adds to the quality and credibility of the information.

Covering the diversity of horticultural crops and techniques is a big challenge that was elegantly solved by the editors. There is a balanced number of chapters devoted to fruits, nuts, ornamentals, vegetables, and also to specific applications of in vitro propagation. This makes this book a broad repository of high quality information on cutting-edge technology.

I congratulate the editors for the opportunity of this initiative. Readers should be happy with this new book on a topic of major horticultural importance that I widely recommend.

*António A. Monteiro*  
*President of the International Society for Horticulture Science (ISHS)*



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## Preface

Micropropagation of horticultural crops, including ornamental plants, is a reliable technology applied commercially worldwide, which allows large-scale plant multiplication, production, and supply of selected plants. Tens of millions of rootstocks and fruit species, gardening and cut-flowers, vegetable plants are produced annually by micropropagation in several European countries, such as Belgium, Holland, Italy, Germany, and France. Moreover, new commercial laboratories have been recently established in Turkey, Greece, Czech Republic, Poland, Hungary, and other Eastern European countries. Outside Europe, micropropagation of horticultural crops is highly advanced and routinely applied commercially, such as in USA, Australia, India, South Africa, China, New Zealand, Argentina, and Brazil. In addition to rapid plant multiplication, tissue culture is largely used also for germplasm conservation, elimination of pathogens, and genetic manipulations.

Micropropagation, however, is highly labor oriented and, thereby, commercial companies are outsourcing plant multiplication activities to low-labor cost areas. Hence, in the technologically advanced countries, the great potential of micropropagation for large-scale plant multiplication can be tapped by cutting down the cost of production per plant, pursued by applying low-cost tissue culture, adopting practices, and optimizing use of equipment and resources to reduce the unit cost of micropropagule and plant production without compromising the quality. Furthermore, the development and rapid multiplication of new ornamental cultivars are required to meet the demand of consumers all year round. The existing and refined protocols for *in vitro* culture, as well as their direct applications in improving and developing new cultivars, regularly supply plant material year round. Moreover, *in vitro* long-term storage of valuable germplasm would immensely provide benefits to both the industry and academic Institutes. The outcome of recent studies carried out in various research laboratories and institutions shows optimized micropropagation protocols for many economically-important species and well-developed *in vitro* techniques, such as thermotherapy and cryotherapy for virus-free production, exploitation of somaclonal variation, long-term shoot culture conservation, and plant rejuvenation.

This book is focused on the recent advances on the micropropagation of several economically important horticultural species. A total of 35 chapters are included, divided into four major sections. Part I contains 13 chapters, covering economically-important fruit and nut species; Part II includes 11 chapters on outdoor/indoor ornamentals and cut-flowers; Part III includes five chapters dealing with vegetables. Each chapter contains a step-wise protocol of micropropagation and a “Notes” section, i.e., an extensive overview based on the personal expertise of contributing authors. Part IV contains six specific reviews on

pivotal topics, such as in vitro rejuvenation, synthetic seed technology, thermotherapy and meristem culture, genetic transformation, flower color somaclonal variation, and cryotherapy of horticultural crops. All submitted manuscripts have been peer-reviewed and revised accordingly.

The readership of the book will be horticulturists, researchers, commercial companies, plant propagators, biotechnologists, and students.

*Florence, Italy*  
*Florence, Italy*  
*Helsinki, Finland*

*Maurizio Lambardi*  
*E. Aylin Ozudogru*  
*Shri Mohan Jain*

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## Contributors

- NASIM AKHTAR • *Department of Biotechnology, GITAM Institute of Technology (Cotton Bhavan), GITAM University, Visakhapatnam, India*
- NANCY M. APÓSTOLO • *Department of Basic Sciences, Plant Tissue Culture Laboratory (CULTEV), National University of Luján, Buenos Aires, Argentina*
- ILDIKÓ BALLA • *Research Institute for Fruitgrowing and Ornamentals, Budapest, Hungary*
- CHRISTINA M. BAVOUGIAN • *Department of Agronomy and Horticulture, University of Nebraska-Lincoln, Lincoln, NE, USA*
- CARLA BENELLI • *IVALSA-Istituto per la Valorizzazione del Legno e delle Specie Arboree, National Research Council (CNR), Sesto Fiorentino, Florence, Italy*
- MARGHERITA BERUTO • *Istituto Regionale per la Floricoltura (IRF), Sanremo, Italy*
- WENLU BI • *Shaanxi Key Laboratory of Molecular Biology for Agriculture, College of Horticulture, Northwest Agriculture and Forestry University, Shaanxi, China*
- ALESSANDRO BISIGNANO • *Istituto Regionale per la Floricoltura (IRF), Sanremo, Italy*
- GABRIELA CLAUDIA CANGAHUALA-INOCENTE • *Laboratory of Plant Developmental Physiology and Genetics, Graduate Program in Plant Genetic Resources, Federal University of Santa Catarina, Florianópolis, Brazil*
- CLARISSA ALVES CAPRESTANO • *Laboratory of Plant Developmental Physiology and Genetics, Graduate Program in Plant Genetic Resources, Federal University of Santa Catarina, Florianópolis, Brazil*
- MAURIZIO CAPUANA • *IGV-Istituto di Genetica Vegetale, National Research Council (CNR), Sesto Fiorentino, Florence, Italy*
- BENEDETTA CHIANCONE • *Dipartimento DEMETRA, Facoltà di Agraria, Università degli Studi di Palermo, Palermo, Italy*
- HOANG XUAN CHIEN • *Tay Nguyen Institute of Biology, Hanoi, Vietnam*
- ZHENHUA CUI • *Shaanxi Key Laboratory of Molecular Biology for Agriculture, College of Horticulture, Northwest Agriculture and Forestry University, Shaanxi, China*
- LIRIO LUIZ DAL VESCO • *Laboratory of Plant Developmental Physiology and Genetics, Graduate Program in Plant Genetic Resources, Federal University of Santa Catarina, Florianópolis, Brazil*
- JEANINE DENOMA • *US Department of Agriculture, Agricultural Research Service, National Clonal Germplasm Repository, Corvallis, USA*
- PHILIPPE DRUART • *Department Life Sciences, Biological Engineering Unit, CRA-W, Gembloux, Belgium*
- EWA DZIEDZIC • *Department of Pomology and Apiculture, Agricultural University, Krakow, Poland*
- CHAOHONG FENG • *Shaanxi Key Laboratory of Molecular Biology for Agriculture, College of Horticulture, Northwest Agriculture and Forestry University, Shaanxi, China*

- MICHAEL P. FULLER • *School of Biomedical and Biological Sciences, University of Plymouth, Plymouth, UK*
- ANTOINE GALIANA • *CIRAD-BIOS, Montpellier, France*
- MARIA ANTONIETTA GERMANÀ • *Dipartimento DEMETRA, Facoltà di Agraria, Università degli Studi di Palermo, Palermo, Italy*
- EDGARDO GIORDANI • *Department of Plant, Soil and Environmental Science (DIPSA), University of Florence, Florence, Italy*
- DOREEN GOH • *YSG Biotech Sdn Bhd., Yayasan Sabah Group, Sabah, Malaysia*
- SANDRA GONÇALVES • *Institute for Biotechnology and Bioengineering (IBB/CGB), University of Algarve, Faro, Portugal*
- MIGUEL PEDRO GUERRA • *Laboratory of Plant Developmental Physiology and Genetics, Graduate Program in Plant Genetic Resources, Federal University of Santa Catarina, Florianópolis, Brazil*
- NGUYEN THANH HAI • *Tay Nguyen Institute of Biology, Hanoi, Vietnam*
- TRUONG THI DIEU HIEN • *Tay Nguyen Institute of Biology, Hanoi, Vietnam*
- NGUYEN PHUC HUY • *Tay Nguyen Institute of Biology, Hanoi, Vietnam*
- GIOVANNI IAPICHINO • *Dipartimento di Sistemi Agro-Ambientali, Università degli Studi di Palermo, Palermo, Italy*
- JOANNA JAGŁA • *The Fruit Experimental Station, Brzezna, Research Institute of Horticulture, Skierniewice, Poland*
- SHRI MOHAN JAIN • *Department of Agricultural Sciences, University of Helsinki, Helsinki, Finland*
- ZORAN JEKNIĆ • *Department of Horticulture, Oregon State University, Corvallis, USA*
- SLADANA JEVREMOVIĆ • *Institute for Biological Research "Siniša Stanković", University of Belgrade, Belgrade, Serbia*
- ERGUN KAYA • *Department of Molecular Biology and Genetics, Gebze Institute of Technology (GYTE), Gebze-Kocaeli, Turkey*
- E.R. JOACHIM KELLER • *Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben, Germany*
- MARTIN KIEFFER • *Centre for Plant Sciences, University of Leeds, Leeds, UK*
- SURINDER KUMAR • *Department of Biotechnology, University of Horticulture and Forestry, Solan, India*
- MAURIZIO LAMBARDI • *IVALSA-Istituto per la Valorizzazione del Legno e delle Specie Arboree, National Research Council (CNR), Sesto Fiorentino, Florence, Italy*
- EZEQUIEL E. LARRABURU • *Plant Tissue Culture Laboratory (CULTEV), Department of Basic Sciences, National University of Luján, Buenos Aires, Argentina*
- LUDIVINE LASSOIS • *Plant Pathology Unit, University of Liege, Gembloux Agro-Bio Tech, Gembloux, Belgium*
- PHILIPPE LEPOIVRE • *Plant Pathology Unit, University of Liege, Gembloux Agro-Bio Tech, Gembloux, Belgium*
- BAIQUAN LI • *Shaanxi Key Laboratory of Molecular Biology for Agriculture, College of Horticulture, Northwest Agriculture and Forestry University, Shaanxi, China*
- JINGWEI LI • *Shaanxi Key Laboratory of Molecular Biology for Agriculture, College of Horticulture, Northwest Agriculture and Forestry University, Shaanxi, China*
- MINGFU LI • *Institute of Animal and Plant Quarantine, Chinese Academy of Inspection and Quarantine (CAIQ), Beijing, China*

- WOJCIECH LITWIŃCZUK • *Faculty of Biology and Agriculture, Department of Plant Physiology and Biotechnology, University of Rzeszów, Rzeszów, Poland*
- BERTA E. LLORENTE • *Plant Tissue Culture Laboratory (CULTEV), Department of Basic Sciences, National University of Luján, Buenos Aires, Argentina*
- LUCIENNE MANSVELT • *ARC Institute for Fruit, Vine and Wine, Stellenbosch, South Africa*
- PABLO MARINANGELI • *Department of Agronomy, Universidad Nacional del Sur, Centro de Recursos Naturales Renovables de la Zona Semiárida (CERZOS), National Research Council (CONICET), Bahía Blanca, Argentina*
- MINAL MHATRE • *Plant Cell Culture Technology Section, Nuclear Agriculture and Biotechnology Division, Bhabha Atomic Research Centre, Mumbai, India*
- MAURIZIO MICHELI • *Department of Agricultural and Environmental Sciences, University of Perugia, Perugia, Italy*
- GHANI MINERVA • *Department of Biotechnology, University of Horticulture and Forestry, Solan, India*
- OLIVIER MONTEUUIS • *CIRAD-BIOS, Montpellier, France*
- LUIS A. MROGINSKI • *Instituto de Botánica del Nordeste (UNNE-CONICET), Facultad de Ciencias Exactas, Corrientes, Argentina*
- SUSAN J. MURCH • *University of British Columbia, Okanagan Campus, Kelowna, Canada*
- NGUYEN BA NAM • *Tay Nguyen Institute of Biology, Hanoi, Vietnam*
- MAR NAVAL • *Instituto Valenciano de Investigaciones Agrarias, Valencia, Spain*
- DUONG TAN NHUT • *Tay Nguyen Institute of Biology, Hanoi, Vietnam*
- ELIF AYLIN OZUDOGRU • *IVALSA-Istituto per la Valorizzazione del Legno e delle Specie Arboree, National Research Council (CNR), Sesto Fiorentino, Florence, Italy*
- BART PANIS • *Laboratory of Tropical Crop Improvement, Division of Crop Biotechnics, Catholic University of Leuven, Leuven, Belgium*
- ROSETE PESCADOR • *Laboratory of Plant Developmental Physiology and Genetics, Graduate Program in Plant Genetic Resources, Federal University of Santa Catarina, Florianópolis, Brazil*
- JOSEPH POSTMAN • *US Department of Agriculture, Agricultural Research Service, National Clonal Germplasm Repository, Corvallis, USA*
- TUI RAY • *Department of Botany, Centre of Advanced Study, University of Calcutta, Kolkata, India; Division of Plant Biology, The Samuel Roberts Noble Foundation, Ardmore, OK, USA*
- PAUL E. READ • *Department of Agronomy and Horticulture, University of Nebraska-Lincoln, Lincoln, USA*
- BARBARA M. REED • *US Department of Agriculture, Agricultural Research Service, National Clonal Germplasm Repository, Corvallis, USA*
- HEBE Y. REY • *Instituto de Botánica del Nordeste (UNNE-CONICET), Facultad de Ciencias Exactas, Corrientes, Argentina*
- ANABELA ROMANO • *Institute for Biotechnology and Bioengineering (IBB/CGB), University of Algarve, Faro, Portugal*
- ROMANO RONCASAGLIA • *Vivai Piante Battistini S.A, Cesena, Italy*
- SATYESH C. ROY • *Department of Botany, Centre of Advanced Study, University of Calcutta, Kolkata, India*

- BARBARA RUFFONI • *Ornamental Plants Research Unit, CRA-FSO, Sanremo, Italy*
- ERMANNIO SACCO • *Ornamental Plants Research Unit, CRA-FSO, Sanremo, Italy*
- PRASENJIT SAHA • *Department of Botany, Centre of Advanced Study, University of Calcutta, Kolkata, India; Plant Biology Division, The Samuel Roberts Noble Foundation, Ardmore, USA*
- MARCO SAVONA • *Ornamental Plants Research Unit, CRA-FSO, Sanremo, Italy*
- PRAVEEN K. SAXENA • *Department of Plant Agriculture, University of Guelph, Ontario, Canada*
- MUKUND SHUKLA • *Department of Plant Agriculture, University of Guelph, Ontario, Canada*
- ANGELIKA SENULA • *Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben, Germany*
- NATAŠA ŠTAJNER • *Centre for Plant Biotechnology and Breeding, University of Ljubljana, Ljubljana, Slovenia*
- ALVARO STANDARDI • *Department of Agricultural and Environmental Sciences, University of Perugia, Perugia, Italy*
- ANGELINA SUBOTIĆ • *Institute for Biological Research “Siniša Stanković”, University of Belgrade, Belgrade, Serbia*
- J. ALAN SULLIVAN • *Department of Plant Agriculture, University of Guelph, Ontario, Canada*
- RONY SWENNEN • *Laboratory of Tropical Crop Improvement, Division of Crop Biotechnics, Catholic University of Leuven, Leuven, Belgium*
- NGUYEN NGOC THI • *Tay Nguyen Institute of Biology, Hanoi, Vietnam*
- PHAM THI MINH THU • *Tay Nguyen Institute of Biology, Hanoi, Vietnam*
- TRAN TRONG TUAN • *Tay Nguyen Institute of Biology, Hanoi, Vietnam*
- INES VAN DEN HOUWE • *Bioversity International Transit Center, Laboratory of Tropical Crop Improvement, Division of Crop Biotechnics, Catholic University of Leuven, Leuven, Belgium*
- SERENA VIGLIONE • *Istituto Regionale per la Floricoltura (IRF), Sanremo, Italy*
- SUGAE WADA • *Department of Horticulture, Oregon State University, Corvallis, USA*
- BIAO WANG • *Shaanxi Key Laboratory of Molecular Biology for Agriculture, College of Horticulture, Northwest Agriculture and Forestry University, Shaanxi, China*
- QIAOCHUN WANG • *Shaanxi Key Laboratory of Molecular Biology for Agriculture, College of Horticulture, Northwest Agriculture and Forestry University, Shaanxi, China*
- REN RUI WANG • *Shaanxi Key Laboratory of Molecular Biology for Agriculture, College of Horticulture, Northwest Agriculture and Forestry University, Shaanxi, China*
- ZHENFANG YIN • *Shaanxi Key Laboratory of Molecular Biology for Agriculture, College of Horticulture, Northwest Agriculture and Forestry University, Shaanxi, China*
- ZHIBO ZHANG • *Shaanxi Key Laboratory of Molecular Biology for Agriculture, College of Horticulture, Northwest Agriculture and Forestry University, Shaanxi, China*

# **Part I**

## **Protocols for Micropropagation of Fruit and Nut Species**

# Chapter 1

## Micropropagation of Pear (*Pyrus* sp.)

Barbara M. Reed, Jeanine DeNoma, Sugae Wada, and Joseph Postman

### Abstract

Elements of micropropagation include establishment of shoot tip cultures, proliferation, rooting, and acclimatization of the resulting plantlets. The wide genetic variation in *Pyrus* makes micropropagation challenging for many genotypes. Initiation of shoots is most successful from forced dormant shoots or from scions grafted onto seedling rootstocks to impose juvenility. Clean shoots are recovered after testing for contaminants at the initiation stage on ½ strength Murashige and Skoog 1962 medium (MS), at pH 6.9 for 1 week or by streaking on nutrient agar. Although pear species and cultivars are cultured on several well-known media, MS is the most commonly used. Our studies showed that multiplication and growth of shoots are best on Pear Medium with higher concentrations of calcium chloride, potassium phosphate, and magnesium sulfate than MS medium and 4.4 μM N<sup>6</sup> benzyladenine. Pear shoots are often recalcitrant to rooting; however, a 5 s dip in 10 mM indole-3-butyric acid or naphthalene acetic acid before planting on basal medium without plant growth regulators is effective for many genotypes. Pear shoots store well at 1–4°C, and can hold for as long as 4 years without reculture. Cryopreservation protocols are available for long-term storage of pear shoot tips. Acclimation of in vitro-rooted or micrografted shoots in a mist bed follows standard procedures.

**Key words:** Micrografting, Micropropagation, Mineral nutrition, Multiplication, *Pyrus*, Shoot culture, Rooting

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## 1. Introduction

Micropropagation techniques are widely used for pear, and protocols are available for the major *Pyrus communis* L. cultivars and for specific cultivars in several other species. General and genotype specific protocols developed empirically for pear are discussed in several reviews (1–3). Most published methods use Murashige and Skoog (MS) (4) basal nutrient medium at full or half strength, or with slight modifications. Lepoivre (LP) (5), Driver–Kuniyuki Walnut (DKW) (6), and Woody Plant Medium (WPM) (7) are also used and differ from MS in types or amounts of calcium and nitrogen.

The major differences in macronutrients among these four basal media are ammonium and nitrate ion concentrations and total ion concentration (8). Recent studies were designed to determine the mineral nutrient requirements for micropropagation of pear shoot tips. Five mineral nutrient factors were created based on MS salts:  $\text{NH}_4\text{NO}_3$ ,  $\text{KNO}_3$ , meso nutrients (Ca-Mg-Cl-Mn- $\text{SO}_4$ - $\text{PO}_4$ ), metals (Zn-Mn-Cu-Co-Mo-B-I), and Fe-EDTA, and each factor was varied over a range of concentrations. Analysis using five genotypes identified the factors with the largest effects: quality (meso nutrients, Fe), leaf spotting/necrosis (meso nutrients), leaf size (meso nutrients), shoot number ( $\text{NH}_4\text{NO}_3$ , Fe), and shoot length (Fe). The analyses of each genotype showed that the factors with the largest effects were similar across the genotypes. Mineral nutrient formulations were identified for groups of genotypes that resulted in significantly better growth and were designated Pear Medium 1 and 2 (Table 1) (9).

Sucrose is the most common carbon source for pear culture. Agar is routinely used as a solidifying agent in the culture medium, but mixtures of agar and gellan gum (Phytigel™ or Gelrite™) (10) or corn starch and gellan gum with a hydric control agent are also used (11).

The cytokinin  $\text{N}^6$  benzyladenine (BA) at 2–10  $\mu\text{M}$  is added in the culture medium for inducing axillary bud growth. Indole-3-butyric acid (IBA), naphthalene acetic acid (NAA) at 0.01–0.05  $\mu\text{M}$ , or no auxin may be included in the multiplication medium. Gibberellic acid ( $\text{GA}_3$ ) is rarely used and may inhibit shoot proliferation or rooting in some cultivars (3, 12).

Pear shoot cultures can be stored at low temperatures (1–4°C) for 1–4 years with excellent results (13–17). We found cold storage best when shoots were grown on MS medium for one cycle before storage on Pear 1 or 2 medium with  $\frac{1}{2}\times$  nitrogen concentrations. Cryopreservation protocols are well developed for pear, and germ-plasm collections are stored as shoot cultures in liquid nitrogen (18, 19).

There is no single best method for rooting micropropagated pear shoots due to the variation in genotype response (10, 20). Methods commonly used to induce rooting include NAA or IBA at 0.1–10  $\mu\text{M}$ , or indole-3-acetic acid (IAA) at 10  $\mu\text{M}$  in MS medium for 1 or more weeks, or a 5 s dip in 10 mM (2,000 mg/L) IBA or NAA (20, 21). These methods have varied results depending upon the genotype and produce rooting frequencies from 28 to 100% for 49 pear species and cultivars (20). A pear micropropagation protocol, in vitro cold storage, and cryopreservation are described.

**Table 1**  
**MS and pear multiplication and storage medium formulations**

Component	MS medium	Pear Medium-1	Pear Medium-2	Pear cold storage
	Conc./L	Conc./L	Conc./L	Conc./L
Stock A— $\text{NH}_4\text{NO}_3$	20 mL	20 mL	20 mL	10 mL
Stock B— $\text{KNO}_3$	20 mL	20 mL	20 mL	10 mL
Stock C <sup>a</sup> — $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	10 mL	15 mL	20 mL	20 mL
Stock D <sup>a</sup> — $\text{KH}_2\text{PO}_4$	10 mL	15 mL	20 mL	20 mL
Stock E <sup>a</sup> — $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	10 mL	15 mL	20 mL	20 mL
Stock F—Sulfates	10 mL	10 mL	10 mL	10 mL
Stock G—Micros	10 mL	10 mL	10 mL	10 mL
Stock H—Iron	10 mL	10 mL	10 mL	10 mL
Thiamine (stock 10 mg/100 mL)	25 mL	25 mL	25 mL	25 mL
Inositol	250 mg	250 mg	250 mg	250 mg
Sucrose	30 g	30 g	30 g	30 g
<sup>a</sup> Based on 1, 1.5 or 2× MS concentrations				
<i>N<sup>6</sup> Benzyladenine*</i>				
1 mg/L (4.4 μM) (stock solution 10 mg/100 mL)	10 mL	10 mL	10 mL	none
<i>Rooting</i>				
(A) 5 s dip in either 10 mM indole-3-butyric acid or 10 mM naphthalene acetic acid dissolved in DMSO, then plant on growth medium without PGR				
(B) 10 μM IBA or IAA in basal medium for 1 week, then on medium without PGR for 3 weeks				
pH	5.7	5.7	5.7	5.7
Agar only or combination of	7 g	7 g	7 g	8 g
Agar	3.5 g	3.5 g	3.5 g	3.5 g
Gelrite	1.45 g	1.45 g	1.45 g	1.75 g

## 2. Materials

### 2.1. Explanting Materials

1. Dormant budwood collected near the end of the dormant season and after chilling requirements have been met. Label clearly and store at 4°C until use. Use this wood for forcing (see below) or grafting onto rootstock. Collect young shoot tips from forces or grafts growing in a sheltered environment

(greenhouse) during the early part of the growing season (see Note 1).

2. Hot water bath: hot water treatment for 10 min at 50°C.
3. Wetting solution (Siltwet, Setre Chemical Co., Memphis, TN) 2 mL/40 L water or liquid dish washing detergent.
4. Cold water bath at about 20°C.
5. Copper dip: NuCop 50 (Albaugh, Inc., Ankeny, IA) at 1 Tbs/gallon or 15 mL/4 L.
6. Florist solution: Floralife (Floralife, Inc., Walterboro, SC).
7. 1-L jars for forcing shoots; one jar for each genotype.

## 2.2. Collection and Surface Sterilization Materials

1. Dissecting scissors.
2. 20% bleach for sterilizing tools as you collect.
3. 100 mL beakers or similar size containers for collecting explants.
4. Cheesecloth squares to cover beakers.
5. Rubber bands to hold cheesecloth in place (Fig. 1a, b).

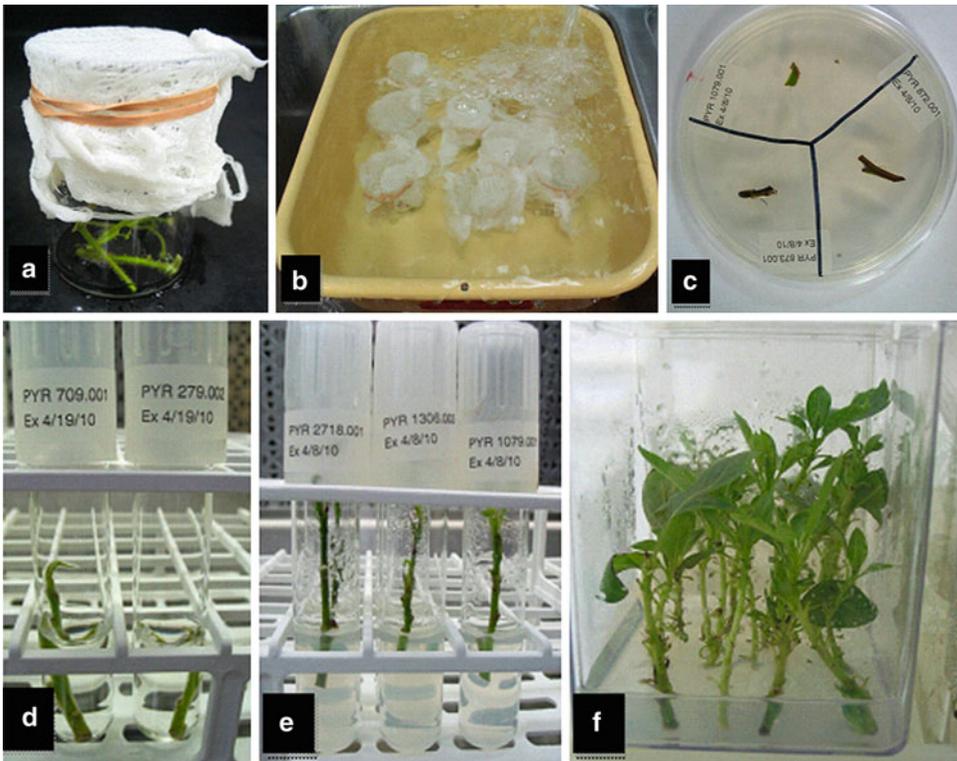


Fig. 1. Initiation and micropropagation of pear shoots. (a) Explants prepared for water rinse. (b) Water rinse before surface sterilization. (c) Contaminant detection on NA plate. (d) Contaminant detection in liquid  $\frac{1}{2}\times$  MS medium. (e) Pear shoots on initiation medium. (f) Multiplication in Magenta box.

6. Tap water.
7. Commercial bleach (high quality, i.e., Clorox brand (6% NaOCl; 5.7% available chlorine)) diluted 1:10 with distilled or deionized water.
8. Tween 20 or other wetting agent.
9. Rotary shaker.
10. Flasks (125 mL) with 75 mL sterile deionized or distilled water; 2 each per genotype for rinses.
11. Tissue culture facilities and tools (Laminar flow bench, scalpel, forceps, tool sterilizer, culture room).

### 2.3. Culture Media

1. Contaminant detection tubes: 16×100 mm capped test tubes with 5 mL of liquid ½ strength MS medium (no PGR, pH 6.9) (see Note 2; Fig. 1c).
2. Contaminant detection medium, nutrient agar (NA) in 9 cm petri plates (Fig. 1d).
3. Multiplication medium (see Tables 1, 2; Note 3; Fig. 1e), 5 mL in 16×100 mm test tubes (gelled).
4. Multiplication medium (Table 1; Fig. 1f), 40 mL in Magenta GA7 cubes (Magenta Corp., Chicago, IL) or glass jars.

**Table 2**  
**Pear medium stock components of MS and Pear medium**

Stock	Compounds	g/L (stock solution)	MS medium (mL)	Pear medium 1 (1.5× CDE) (mL)	Pear medium 2 (2× CDE) (mL)
Stock A	NH <sub>4</sub> NO <sub>3</sub>	82.5	20	20	20
Stock B	KNO <sub>3</sub>	95.0	20	20	20
Stock C	CaCl <sub>2</sub> ·2H <sub>2</sub> O	44.0	10	15	20
Stock D	KH <sub>2</sub> PO <sub>4</sub>	17.0	10	15	20
Stock E	MgSO <sub>4</sub> ·7H <sub>2</sub> O	37.0	10	15	20
Stock F	MnSO <sub>4</sub> ·H <sub>2</sub> O	1.66	10	10	10
	CuSO <sub>4</sub> ·5H <sub>2</sub> O	0.0025			
	ZnSO <sub>4</sub> ·7H <sub>2</sub> O	0.860			
Stock G	KI	0.083	10	10	10
	CoCl <sub>2</sub> ·6H <sub>2</sub> O	0.0025			
	H <sub>3</sub> BO <sub>3</sub>	0.620			
	Na <sub>2</sub> MoO <sub>4</sub> ·2H <sub>2</sub> O	0.025			
Stock H <sup>a</sup>	FeSO <sub>4</sub> ·7H <sub>2</sub> O	2.78	10	10	10
	EDTA·Na <sub>2</sub>	3.724			

<sup>a</sup>Heat stock H with stirring until golden, then cool and store in a dark bottle

5. Cold storage medium (see Table 1; Note 4) without growth regulators.
6. Rooting medium (see Table 1; Note 5), 40 mL in Magenta GA7 cubes or glass jars.
7. Dimethylsulfoxide (DMSO) (~100 mL) for dissolving auxins if using the dip method.

#### **2.4. Cold Storage of Shoot Cultures**

1. Shoot cultures ~5 cm tall (Fig. 3a).
2. Storage containers: Semipermeable plastic tissue culture bags (StarPac bags, Garner Enterprises, TX), test tubes, boxes or jars.
3. Storage medium without growth regulators (see Table 1).
4. Cold acclimation chamber ( $-1^{\circ}\text{C}$  16 h dark/ $22^{\circ}\text{C}$  8 h light) (see Note 4).
5. Cold storage facility (incubator or cold room at  $1-4^{\circ}\text{C}$ ).

#### **2.5. Acclimation Materials**

1. Sterile potting mix or preformed medium (Oasis cubes, Smithers-Oasis Co., Cuyahoga Falls, OH).
2. Mist bed or clear plastic cups or glass jars to cover plants.
3. Seedling rootstocks for micrografting if rooting is not successful.

#### **2.6. Micrografting Materials**

1. Potted pear seedlings that have the same stem diameter as the micropropagated shoots (see Note 6).
2. Glass jars or plastic cups or bags for covering the plants after grafting.
3. Grafting rubbers and razor blade, scalpel or sharp knife.

#### **2.7. Cryopreservation Materials**

1. Cold acclimated plants for shoot tips. (1–4 weeks at  $-1^{\circ}\text{C}$  for 16 h/ $22^{\circ}\text{C}$  for 8 h) (see Note 4).
2. Petri dishes of pretreatment medium, one for each treatment group (MS medium with 5% DMSO and 8% agar).
3. Fine dissecting tools and a binocular low-power microscope for dissection.
4. Sterile beakers or small petri dishes for holding cryoprotectant and liquid media.
5. Sterile 2 mL transfer pipettes.
6. Sterile filter flasks for cryoprotectant sterilization.
7. Sterile filter paper strips for draining shoot tips.
8. Loading solution of 2 M glycerol and 0.4 M sucrose in MS liquid medium.
9. Rinsing solution of liquid MS medium with 1.2 M sucrose at pH 5.8.

10. Recovery medium (use standard growth medium without auxin in small petri dishes or multiwell plates).
11. Cryotubes.
12. Cryotube holder frozen in a block of ice (or use crushed ice).
13. Dewar with liquid nitrogen.

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### 3. Methods

Micropropagation of pear is somewhat genotype dependent; however, a few general principles apply. To produce healthy cultures, select healthy source material and treat it to exclude insects and insect eggs (wash or apply a dormant oil treatment). Collect only actively growing, disease-free shoots; follow the appropriate initiation and growth procedures (see Note 1). After collecting shoots, test the explants for bacterial and fungal contamination to provide contamination-free shoots for the multiplication phase (see Note 2). Finally, determine the appropriate growth medium (see Note 3), cold storage conditions (see Note 4), rooting protocol (see Notes 5 and 6) and acclimation procedures to allow the production of healthy rooted plantlets.

#### **3.1. Preparation and Sterilization of Culture Media**

##### *3.1.1. Bacterial Detection Media*

1. Liquid MS detection medium.
  - (a) Prepare  $\frac{1}{2}$  strength MS medium without PGR, pH 6.9, and dispense 5 mL into 16×100 mm test tubes (see Note 2).
  - (b) Partially place caps on tubes (so steam can escape during autoclaving). Push the caps down completely after autoclaving.
  - (c) Autoclave at 121°C for 20 min (118 kPa pressure).
2. Nutrient Agar detection medium.
  - (a) Prepare an enriched Nutrient Agar (NA) adding yeast extract (1 g/L) and glucose (10 g/L) according to the package directions (see Note 2).
  - (b) Cool slightly after autoclaving and pour into 9 mm Petri plates in the laminar flow hood. Let plates cool with lids partially open for 45 min to evaporate excess moisture before storing.
  - (c) Plates can be stored upside down in a plastic bag for up to 2 months at room temperature.

##### *3.1.2. Pear Culture and Storage Medium*

1. Prepare Pear Multiplication Medium from the formulations in Table 1 (see Note 3).
  - (a) In an appropriate sized beaker, add distilled or deionized water up to  $\frac{1}{2}$  the final medium volume (i.e., 500 mL for 1 L medium).

- (b) Add the mineral salts, vitamins, sucrose, and plant growth regulators, stirring after each addition until all are dissolved.
  - (c) Bring to the final volume with distilled or deionized water, mix well and adjust the pH to 5.7 with NaOH or H<sub>3</sub>PO<sub>4</sub>.
  - (d) Add gelling agent (see Table 1), heat until gelling agent is fully dissolved and dispense into autoclavable containers. Use 5 mL in each 16×100 mm tube for shoot initiation and 40 mL in each Magenta Box for multiplication (GA7, Magenta Corp., Chicago, IL). Partially place caps on tubes (so steam can escape during autoclaving) and push them down completely after autoclaving.
  - (e) Autoclave at 121°C for 20 min (118 kPa pressure).
  - (f) Store the medium in a clean area and use within 2 weeks.
2. Prepare Cold Storage Medium from the formulation in Table 1.
    - (a) Use stock plants grown for one cycle on MS medium.
    - (b) If using semipermeable tissue culture bags, increase agar by 1 g/L over that in multiplication medium.
    - (c) Storage medium should not contain PGRs.
    - (d) Dispense into containers and autoclave.
    - (e) Plant 5 cm long shoots and seal bags.
    - (f) Grow for 1 week in the growth room and 1 week in cold acclimation (see Note 4).
    - (g) Store at 1–4°C with 12 h of low light, or in the dark.
    - (h) Check the condition of the plants at 4 month intervals and repropagate as needed.
  3. Prepare Pear Rooting Medium from the MS formulation in Table 1 (see Note 5).
    - (a) For rooting with the quick dip method, prepare standard medium without growth regulators (see Note 5).
    - (b) Have DMSO available for dissolving auxins.
    - (c) For rooting in medium with auxin, make MS medium as above with the growth regulators indicated in Note 5.

### **3.2. Plant Source Material and Surface Sterilization**

#### **3.2.1. Grafting Budwood**

1. Collect budwood at the end of the dormant season after the chilling requirement has been met and store at 4°C until use.
2. Graft onto seedling rootstocks with a cleft graft in late winter or early spring.
3. Grow the grafted plants in a protected environment (such as a greenhouse).
4. When the new growth is 5–10 cm, collect shoots for explants and follow surface sterilization procedures below.

### 3.2.2. Forcing Budwood

1. Collect budwood at the end of the dormant season, after the chilling requirement has been met and store at 4°C until use.
2. Remove budwood from cold storage and gently scrub with soft brush under cool running tap water.
3. Submerge in a 50°C circulating water bath with a wetting agent (2 mL/40 L) for 10 min.
4. Transfer immediately to a cool (~20°C) tap water bath for 10 min.
5. Rinse under running tap water and allow to slightly dry.
6. Dip in copper solution (Nu Cop 50 at 1 Tbs/gal or 15 mL/4 L).
7. Dry completely; wear gloves when handling copper-treated plants.
8. Mix FloraLife at 10 g/L (or as indicated on label) using distilled or deionized water and fill 1 L jars to half full.
9. Trim end of each stem with a diagonal cut and place shoots in jar.
10. Dip pruning shears in 20% bleach to sterilize between each cut.
11. Place treated budwood in jars in a greenhouse or warm windowsill.
12. Change the florist solution weekly, recut the stems and remove any flower buds.

When buds have expanded and grown to 5–10 cm, excise a 3–5 cm shoot tip for surface sterilization.

### 3.2.3. Collection and Surface Sterilization of Explants

1. Cut shoots from the plant with clean scissors. Dip scissors in 20% bleach solution between each cut. Cut off all leaves (do not pull them to remove).
2. Place cut shoots into clean 100 mL beaker, cover the beaker with cheesecloth and secure it with a rubber band (Fig. 1a).
3. Place the beakers in a plastic dishpan under running tap water for 10 min. Remove from the water and drain. Remove cheesecloth (Fig. 1b).
4. Cover explants in beaker with bleach solution—10% bleach in deionized water with 3 drops of Tween 20 L.
5. Place on an orbital shaker at approximately 25–30 rpm for 10 min.
6. Move beakers to laminar flow hood and transfer shoots to flasks of sterile water for 2 min, swirl; transfer to fresh sterile water for 2 min.

### 3.2.4. Explant Trimming and Contaminant Detection

1. Place shoot on a clean sterile surface and trim the base. Sterilize the blade between each cut and place each shoot on a new sterile surface.

2. Submerge each shoot in a tube of liquid  $\frac{1}{2}$  strength MS bacterial detection medium, cap and label (Fig. 1c).
3. Place tubes in the growth room under low light for 1 week. Discard any shoot tips in tubes that become cloudy or contaminated.
4. If liquid medium is clear after 1 week, remove the explant from tube, use a clean sterile surface and blade to trim the base of each shoot; place the shoot tip in a tube of solid propagation medium. Use the trimmed base to streak across the bacterial detection plate, leaving a piece of it on the NA medium. Number the plate to match the shoot and keep this plate for 3 weeks. Watch for bacterial or fungal growth (Fig. 1d, e).
5. After 3 weeks, if the NA plate is clear, move the explants to multiplication medium in a container with other explants of the same genotype (Fig. 1f). Discard any shoots with contaminated NA plates.

### **3.3. Multiplication**

1. Divide shoots into 2 or 3 node segments (~3–5 cm) and move to fresh multiplication medium every 3 weeks.
2. Test for bacterial contamination by streaking plant on an NA plate as needed (Fig. 1d).
3. Plant growth regulators: N<sup>6</sup> benzyladenine (BA) 1 mg/L (4.4  $\mu$ M).
4. Growth conditions: Grow at 25°C with 16 h light at 70–100  $\mu$ E/m<sup>2</sup>/s measured at the top of the culture container.

### **3.4. Rooting**

1. Allow shoots to elongate to ~5 cm before rooting.
2. Trim the base of each shoot and place on rooting medium.
3. For dip method, trim the base, dip for 5 s in a 10 mM IBA or NAA solution (dissolved in DMSO), and plant on medium without growth regulators (see Note 5).
4. Let roots develop until they are 3–5 cm long.
5. If they do not root in vitro dip in commercial rooting solution and plant directly in the greenhouse.
6. If rooting is not possible, micrograft the shoots onto seedling rootstocks of a similar diameter (see Note 6; Fig. 2).

### **3.5. Acclimation**

1. Two days before transfer to the greenhouse, loosen the lid on the container to allow more airflow.
2. Remove rooted shoots from the agar medium and rinse off the agar.
3. Plant in a light potting mix and place in a mist bed or cover with a transparent plastic bag, clear plastic cup or glass jar.
4. Move to greenhouse bench after 2–4 weeks.



Fig. 2. Micrografting pear shoots. (a) Micrografting pear shoots onto seedling rootstock. (b) Acclimation of grafted shoots.

### 3.6. Cold Storage of Shoot Cultures

1. Pear shoots can be cold acclimated and stored at 1–4°C for several years (1–4) without transfer to fresh medium.
2. Grow pear cultures on MS medium for one growth cycle (3 weeks).
3. Transfer shoots to heat-sealed plastic tissue culture bags containing fresh storage medium (see Table 1). Storage medium is Pear 2 medium with  $\frac{1}{2}$  MS nitrogen and an additional 1 g/L agar and no PGRs (Fig. 3).
4. Grow for 1 week in growth room conditions.
5. Grow for 1 week in cold acclimation conditions (see Note 4).
6. Store at 1–4°C with 12 h of low light ( $10\text{--}20 \mu\text{E}/\text{m}^2/\text{s}$ ) or in the dark.
7. In general storage of pear cultures averages 2–3 years. Length of storage varies by genotype, so it is necessary to evaluate the stored plants at 4–6 month intervals and repropagate as needed (see Fig. 3; see Note 7).

### 3.7. Cryopreservation (Vitrification Procedure)

1. Two days before cryopreservation dissect 0.8 mm shoot tips from cold acclimated shoot cultures and place on pretreatment medium (standard growth medium with 5% DMSO and 1 g/L more agar).
2. Place in cold acclimation incubator for 2 days ( $-1^\circ\text{C}$  for 16 h/ $22^\circ\text{C}$  for 8 h).
3. Make the cryoprotectant (PVS2) (22). Mix 30% Glycerol, 15% Ethylene Glycol, 15% DMSO, 0.8 M sucrose in liquid MS medium, and adjust to pH 5.8. Filter sterilize before use and chill at  $\sim 4^\circ\text{C}$  until needed. Mix and filter sterilize cryoprotectant the same day as the experiment; do not store it.
4. Add 1 mL of loading solution (2 M glycerol and 0.4 M sucrose in MS medium) to cryotubes.



Fig. 3. Cold storage of pear shoots in tissue culture bags on cold storage medium with  $\frac{1}{2}$  MS nitrogen, more agar than regular medium and no PGRs. (a) Newly planted shoots. (b) Shoots after 2 years storage at 4°C with 12 h low light. (c) Shoots after 4 years storage at 4°C with 12 h low light.

5. Remove shoot tips from pretreatment medium and add to loading solution in cryotubes; hold for 20 min on ice.
6. Vitrification procedure
  - (a) Fill small dewar with liquid nitrogen (LN).
  - (b) After 20 min remove loading solution from cryotubes using a sterile 2 mL transfer pipet and being careful to leave the shoot tips in the tube.
  - (c) Add 1 mL PVS2 to the tubes. START A TIMER. Careful timing is critical as excess exposure to PVS2 is toxic to the shoot tips.
  - (d) Hold shoot tips in PVS2 cryoprotectant at 0°C for a total of 20 min from initial contact with PVS2 (see Note 8).
  - (e) Before 20 min, gently close caps on cryotubes (do not over tighten or it will allow LN to leak into the tubes).
  - (f) Submerge tubes in LN one at a time using forceps. Hold tube under the surface for 15 s before releasing.
7. Rewarming procedure
  - (a) Transfer 1 or 2 vials from LN to 45°C water for 1 min with stirring.
  - (b) Move to 25°C water for 1–2 min. (sterile water or clean tap water may be used).
  - (c) Dry the outside of the tubes before opening.
  - (d) Quickly remove half the PVS2 using a 2 mL sterile transfer pipet.
  - (e) Immediately add 1.2 M sucrose rinsing medium to the top of the tube.
  - (f) Remove the solution in the cryotube and replace with rinsing medium two times.
  - (g) Transfer the shoot tips to filter paper and then place recovery medium.
  - (h) Grow recovering shoot tips on an unlit shelf or in the dark for 1 week before moving them to a shelf with light.
8. Several other techniques are also available for the cryopreservation of micropropagated shoot tips (18, 19).

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## 4. Notes

1. In all tissue culture procedures, the quality and health of mother plants as a source of explants are of prime importance. Always choose healthy, actively growing shoots without any disease symptoms. Keep the parent plant in good condition and collect

shoots during the active growing season in most cases. When forcing dormant shoots, be careful to change the water weekly and recut the stems with the shears cleaned between each stem. Grafted scions retain their juvenile characteristics for up to 2 years; explants from older grafted plants are less successful and are more likely to have bacterial contamination.

2. It is best to check for contamination early in the process to avoid contamination carry over from one plant to others (Fig. 1c–e). Our initiation protocol is to place each explant in a tube of liquid detection medium for 1 week ( $\frac{1}{2}\times$  MS at pH 6.9). If the tube becomes cloudy plant is discarded. If the tube is clear, then the base of the explant is streaked on an NA plate and the plant is transferred to a tube of solid growth medium for 3 weeks. The NA plate is monitored for additional contamination and, if contamination is observed, that shoot is discarded. If the plate is clear at 3 weeks then the clean shoots can be grouped together in a box or jar. NA plates can also be used later in the process to check for latent contaminants.
3. Initiation should be on Pear Medium-1 (see Table 1). Multiplication medium varies by genotype. We suggest starting with Pear Medium-1 and changing to Pear Medium-2 if the growth is not rapid.

The following genotypes were tested with the two formulations and the best growth was seen with: Pear Medium-1: *Pyrus communis* cvs. Horner 51, Luscious, Ayers, Winter Nelis, Pyrodwarf rootstock, *Pyrus pyrifolia* (HB) (Burm. f.) Nakai and cvs. Hosui, Seuri Li, Sion Sz Mi, and *P.* hybrid selection NY 10353. Pear Medium-2: *P. communis* cvs. Bartlett, Old Home x Farmingdale 87, UBILEEN Gift, *Pyrus calleryana* cv. Capital, *Pyrus ussuriensis* cvs. Hang Pa Li, Harbin, *Pyrus cordata* (Turkey), and *Pyrus koelmii*.

4. Cold acclimation of shoots is used to increase the length of cold storage for pear shoot cultures. Micropropagated temperate plants quickly respond to cold acclimation with alternating temperatures. One or 2 weeks at 22°C with 8 h light (10  $\mu$ E m/s) and -1°C with 16 h dark is very effective for acclimation of several genera and results in longer cold storage for many genotypes (17, 23, 24).
5. Forty-nine pear species and cultivar accessions were tested for rooting at NCGR (20). Auxin treatment was required in most cases. A 15 s dip in 10 mM IBA solution (IBA dissolved in DMSO) was effective for 18 accessions; 12 pear genotypes rooted on medium with 10  $\mu$ M IBA for 1 week followed by medium without PGRs for 3 weeks. Rooting with IBA was poor for 28 accessions but a 15 s dip in 10 mM NAA (dissolved in DMSO) was effective for 8 genotypes. Ten pears rooted on 10  $\mu$ M IAA medium either in the dark or at 30°C.

Species selections were the most commonly recalcitrant to rooting, while 25 of 32 cultivars and three species rooted on one of the treatments tested.

6. Micro grafts may be used for the transfer of shoot cultures to the greenhouse. *In vitro* shoots (1.5–3.5 cm) can be removed from the culture container and use as scions for a modified cleft graft (25). The rootstock can be either green and succulent, or woody, but should be similar or slightly larger in diameter to the scion shoot. The rootstock is cut off at a convenient distance above the soil line and a vertical cut is made with a single-edge razor blade about 0.5 cm deep down the center of the rootstock stem. A “V” shaped cut is made at the base of the scion, and the scion is inserted into the cut rootstock. The graft is wrapped with small piece of Parafilm that has been slightly stretched prior to wrapping (Fig. 2a). The completed graft must be covered with a bottle (Fig. 2b) or placed under mist for about 3 weeks to maintain high humidity while the graft union forms. Afterwards, the bottle is gradually removed to acclimate to greenhouse conditions (26).
7. Medium with low nitrogen is critical for storage of the pear shoots. Our studies indicate that low nitrogen and lower nutrients in the final growth cycle before storage, followed low nitrogen and higher nutrients in the storage medium, provides the optimal storage duration for pear shoots.
8. PVS2 vitrification solution is used to dehydrate the cells so water remaining in the cells will vitrify (turn to a glass) on contact with LN. Over exposure to PVS2 will kill the cells from over dehydration while under exposure will kill the cells because crystals will form in the cells. Determining the optimum exposure is important for success of cryopreservation procedures.

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## In Vitro Propagation of Jojoba

Berta E. Llorente and Nancy M. Apóstolo

### Abstract

Jojoba (*Simmondsia chinensis* (Link) Schn.) is a nontraditional crop in arid and semi-arid areas. Vegetative propagation can be achieved by layering, grafting, or rooting semi-hardwood cuttings, but the highest number of possible propagules is limited by the size of the plants and time of the year. Micropropagation is highly recommended strategy for obtaining jojoba elite clones. For culture initiation, single-node explants are cultivated on Murashige and Skoog medium (MS) supplemented with Gamborg's vitamins (B5), 11.1  $\mu\text{M}$  BA ( $\text{N}_6$ -benzyl-adenine), 0.5  $\mu\text{M}$  IBA (indole-3-butyric acid), and 1.4  $\mu\text{M}$  GA<sub>3</sub> (gibberellic acid). Internodal and apical cuttings proliferate on MS medium containing B5 vitamins and 4.4  $\mu\text{M}$  BA. Rooting is achieved on MS medium (half strength mineral salt) amended with B5 vitamins and 14.7  $\mu\text{M}$  IBA during 7 days and transferred to develop in auxin-free rooting medium. Plantlets are acclimatized using a graduated humidity regime on soil: peat: perlite (5:1:1) substrate. This micropropagation protocol produces large numbers of uniform plants from selected genotypes of jojoba.

**Key words:** Micropropagation, Plant growth regulators, Plant tissue culture, *Simmondsia chinensis*

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## 1. Introduction

*Simmondsia chinensis* (Link) Schn. (jojoba) is a nontraditional crop in arid and semi-arid areas. It is naturally well adapted to saline soils and high temperature environmental conditions (1). Jojoba seeds store lipids in the form of liquid wax that makes up 40–60% of their dry weight. This wax and its derivatives have potential for wider applications in cosmetics, pharmaceuticals, lubricants, extenders, and antifoaming agents (2, 3). There is an increased interest in the agricultural production of jojoba and more promising experience has accumulated every year respecting cultivation requirements, planting densities, management practices, productivity, propagation techniques, and genetic improvement (1, 2).

Jojoba is dioecious and unable to initiate sexual reproduction before flowering (usually 2–4 years from germination) (1).

Propagation by direct seeding has genetic heterogeneity and half of the seedlings are males. However, 8–10% males are necessary for pollination (4). Setting up a plantation with asexual propagules is more expensive than with seed, and saves time in replanting plants as well as crop produced of known sex and lineage. Vegetative propagation can be achieved by layering, grafting, or rooting semi-hardwood cuttings, but the highest number of propagules is limited by plant size and time of year (2, 4).

Micropropagation of elite individuals exploits totipotency of plant cells and offers a promising means of commercial mass production of pathogen-free superior clones. *In vitro*-derived jojoba plants grow more vigorously than both seedlings and rooted cuttings, and are significantly larger after the first year of growth (1, 2, 4, 5).

Some protocols for *in vitro* culture of jojoba are known. There are reports of jojoba somatic embryogenesis from zygotic embryo (6, 7) and leaf explants (7, 8). A number of researchers have described *in vitro* culture of single-node explants using both axillary and apical buds (2, 5, 9–15).

Shoots exhibited differential morphogenic behavior under the influence of growth regulators and adjuvants during *in vitro* propagation (9–11). Variations in the response of the explants have been observed in terms of percentage of shoot regeneration, proliferation rate, shoot length, callus presence, and rooting behavior (9–11). Some genotypes exhibited 75% root formation while others displayed scarce rooting in medium with 6  $\mu\text{M}$  indole-3-butyric acid (IBA) (9) and the pulse treatment of 50  $\mu\text{M}$  IBA caused 44–67% *in vitro* rhizogenesis of various genotypes tested (11). On the other hand, no significant difference in bud initiation, rooting and survival in greenhouse was observed in some genotypes studied (14).

A basic protocol for jojoba micropropagation suitable as a starting point for all genotypes is described in this chapter. However, it is recommended to develop an optimized protocol for each genotype because *in vitro* response of each clone can be unpredictable.

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## 2. Materials

1. Systemic and contact fungicidal solution (4% Metalaxil-M + 64% Mancozeb, Fich M™, Cheminova Agro de Argentina S.A.) diluted to 0.2% with tap water.
2. Sodium hypochlorite solution (commercial bleach solution 5.5 g/L active chlorine).
3. Surfactant Tween 20 solution 0.1% (v/v).

4. Autoclaved distilled water, 150 mL aliquots in 250 mL screw-capped bottles.
5. Doubled distilled water.
6. Basal medium (BM) contained Murashige and Skoog salts (MS) (16) and B5 Gamborg's vitamins (17) (Table 1).
7. d-sucrose pure.
8. Agar (e.g., plant cell culture tested from Sigma-Aldrich).
9. N<sub>6</sub>-benzyl-adenine (BA).
10. Indole-3-butyric acid (IBA).

**Table 1**  
**Plant culture medium**

<b>MS salts (16)</b>	<b>mg/L<sup>a</sup></b>	<b>mg/L<sup>b</sup></b>
KNO <sub>3</sub>	1,900	950
NH <sub>4</sub> NO <sub>3</sub>	1,650	825
MgSO <sub>4</sub> ·7H <sub>2</sub> O	370	185
KH <sub>2</sub> PO <sub>4</sub>	170	85
CaCl <sub>2</sub> ·2H <sub>2</sub> O	440	220
H <sub>2</sub> BO <sub>3</sub>	6.2	3.1
MnSO <sub>4</sub> ·H <sub>2</sub> O	16.9	8.5
ZnSO <sub>4</sub> ·7H <sub>2</sub> O	8.6	4.3
Na <sub>2</sub> MoO <sub>4</sub> ·2H <sub>2</sub> O	0.25	0.125
CuSO <sub>4</sub> ·5H <sub>2</sub> O	0.025	0.012
CoCl <sub>2</sub> ·6H <sub>2</sub> O	0.025	0.012
KI	0.83	0.415
FeSO <sub>4</sub> ·7H <sub>2</sub> O	27.8	13.9
Na <sub>2</sub> EDTA·2H <sub>2</sub> O	37.3	18.6
Sucrose	30,000	20,000
<b>Gamborg's B5 vitamins (17)</b>		
Thiamine·HCl	10	10
Pyridoxine·HCl	1	1
Nicotinic acid	1	1
Myo-inositol	100	100
pH	5.8	5.8

<sup>a</sup>Initiation and proliferation media

<sup>b</sup>Rooting medium

11. Gibberellic acid ( $GA_3$ )
12. KOH solutions 0.1, 0.5, and 1.0 M.
13. HCl solutions 0.1, 0.5, and 1.0 M.
14. Flat-bottom glass tubes 55 mL capacity ( $2.7 \times 10$  cm).
15. Potting media, consisting of soil (a horizon from a typical Argiudol), peat (commercial turf Terrafertil™) and perlite in a 5:1:1 by volume.
16. Glass flasks 350 mL ( $6.4 \times 11$  cm).
17. Screw cap tubes.
18. Sterile Petri dishes (9 cm in diameter).
19. Beaker  $\times 1,000$  mL.
20. Scalpels, forceps, spirit burner to flame sterilize instruments.
21. Pruning shears.
22. Laboratory transparent film (30–150  $\mu$ m in thickness).
23. Transparent plastic bags.
24. Plastic pots (8 and 12 cm in diameter).
25. Magnetic stirrer, magnetic bar.
26. Microwave cooker.
27. Laminar flow bench.
28. Culture room or growth chamber.
29. Glasshouse.

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### 3. Methods

This protocol has six main stages: (1) preparation and sterilization of culture media; (2) surface sterilization of plant source material; (3) establishment of axenic cultures of stem nodal explants (culture initiation); (4) shoot proliferation; (5) rooting of in vitro shoots; and (6) acclimatization of regenerated shoots in ex vitro conditions.

#### **3.1. Preparation and Sterilization of Stock Solutions and Culture Media**

1. Prepare BM medium in doubled distilled water supplemented with 3% (w/v) sucrose. Store at 4°C.
2. Prepare IBA and  $GA_3$  stock solutions by dissolving the powder in a few drops of KOH 0.1 M and add doubled distilled water to make up the volume to 1.0 mM. Store at -20°C.
3. Prepare BA stock solution: Dissolve powder in a few drops of HCl 0.1 M and add doubled distilled water to make up the volume to 1.0 mM. Store at -20°C.

4. Initiation medium: Use BM, supplemented with 11.1  $\mu\text{M}$  BA, 0.5  $\mu\text{M}$  IBA, and 1.4  $\mu\text{M}$  GA<sub>3</sub> from stock solutions.
5. Proliferation medium: To BM add 4.4  $\mu\text{M}$  BA from stock solution.
6. Rooting medium (RM): Reduce concentration of MS mineral salts to half and sucrose to 2% (w/v).
7. Root induction: Transfer shoots to RM containing 14.7  $\mu\text{M}$  IBA and for root development use hormone-free RM medium.
8. Adjust the pH of the media to 5.8 with HCl or KOH (1.0, 0.5, or 0.1 M).
9. Add agar 0.7% (w/v) for the initiation and proliferation media and 0.6% (w/v) for rooting media. Dissolve by microwaving.
10. Dispense the media into suitable containers, 15 mL aliquots into 55 mL capacity flat-bottom glass tubes for the shoot initiation and rooting medium, or 60 mL aliquots into 300 mL glass flasks for shoot proliferation.
11. Sterilize the media by autoclaving at 121°C for 20 min (101 kPa steam pressure).
12. Store the autoclaved media at room temperature in the dark for a maximum of 20 days.

### **3.2. Surface**

#### ***Sterilization of Plant Source Material***

1. Choose healthy plants of known sex.
2. Spray the parental stock plants with fungicidal solution 48 h before initiating tissue culture (see Note 1).
3. Cut stem nodal sections (3–6 nodes from apex) of actively growing adult jojoba plants (see Note 2).
4. Cut single-node segments with two axillary buds or one apical bud from stem nodal section.
5. Wash nodal sections for 2 h under running tap water, predisinfect with 2 g/L fungicide (Fich M™); and agitate in a sterile beaker on a magnetic stirrer for 60 min (see Note 3).
6. Disinfect stem segments by immersion in 20% commercial sodium hypochlorite solution, added with two drops of surfactant, and agitate with a magnetic stirrer for 30 min.
7. Sterilize the laminar flow surface by 70% ethanol before use.
8. Rinse in sterile distilled water three times to remove the sterilizing agent under laminar flow.
9. Store in sterile distilled water until culture initiation (no longer than 1 h), under laminar flow.

### **3.3. Environmental Conditions for Culture**

1. Incubate in vitro cultures in a growth room at 24 ± 2°C under Phillips fluorescent daylight tubes (30–40  $\mu\text{mol}/\text{m}^2\text{s}$ ) with a 16 h photoperiod.

2. At the acclimatization stage, incubate the regenerated *in vitro* plants in a growth room at  $24 \pm 2^\circ\text{C}$ , with a 16 h photoperiod and  $40\text{--}50 \mu\text{mol}/\text{m}^2/\text{s}$  light intensity.
3. Glasshouse conditions oscillate from 0 to  $300 \mu\text{mol}/\text{m}^2/\text{s}$  light intensities,  $20\text{--}40^\circ\text{C}$  temperature and 95–40% relative humidity (night to day, respectively).

### **3.4. Initiation of Culture**

1. Sterilize and flame all instruments and laminar flow surface.
2. Cut ends and white portions of the each axenic explant.
3. Eliminate apical cross section of the leaf blades (see Note 4).
4. Firmly insert the base of node segment into 15 mL initiation medium in a flat-bottom tube (see Note 5). A minimum of 30 explants per genotype is recommended for initiation (see Fig. 1a).
5. Seal the tube with transparent film.
6. Incubate in the growth room (see Fig. 1b, d).
7. Suggested parameters to evaluate: frequency rate of sprouting shoot, number of regenerated shoots per explant, shoot length, presence of callus, and number of hyperhydric plantlets for each genotype.
8. When the shoots grow, transfer to the proliferation medium (4–6 weeks).

### **3.5. Shoot Proliferation**

1. Sterilize and flame all instruments and laminar flow before use.
2. Select well developed shoots from initiation culture (see Fig. 1d).
3. Remove hyperhydric, malformed, oxidized and dead tissues (see Note 6). Excise callus and basal part of regenerated shoots (see Note 7).
4. Cut single-node segments with two axillary buds or one apical bud and transfer them to 350 mL flasks (8–10 shoots per flask) with 60 mL sterile proliferation medium (see Fig. 1e).
5. Seal the tube with plastic wrap (see Note 8).
6. Incubate shoots in a growth room and regularly subculture at 4–5 week interval to the same fresh medium for continued proliferation (see Fig. 1f).
7. Suggested parameters to evaluate: proliferation rate (relationship between final and initial node numbers), number of leaves, shoot length, percentage of hyperhydric shoots, and callus development.

### **3.6. Rooting**

1. Sterilize and flame all instruments and laminar flow before use.



Fig. 1. Jojoba micropropagation. (a–d) Initiation culture; (e–g) Shoot proliferation; (h) In vitro rooting; (i, j) Acclimatization; (k) Micropropagated plants in glasshouse. Bars: a–e, g, h, j: 1 cm; f, i: 2 cm; k: 5 cm.

2. Select elongated shoots (4–5 cm) from proliferation culture (see Fig. 1g).
3. Remove hyperhydric, malformed, oxidized, and dead tissues. Excise basal callus (see Notes 6 and 7).
4. Insert the base of 3- to 5-node shoots into flat-bottom tube with 20 mL aliquots of rooting induction medium RM supplemented with 14.7  $\mu\text{M}$  IBA.
5. Seal the tube with plastic wrap.
6. Incubate cultures during 7 days for root induction (“pulse treatment”).
7. Subculture shoots in a flat-bottom tube with 20 mL aliquots of RM root development medium devoid of plant growth regulators.
8. Seal the tube with plastic wrap.
9. After 6–8 weeks of culture, the regenerated shoots will develop root systems consisting of some long roots (see Fig. 1h).
10. Suggested parameters to evaluate: rooting percentage, root number per shoot and quality, root length, dry weights of shoots and roots, root surface area, time until the appearance of the first root (precocity) for each shoot and morphological features (see Notes 9 and 10).
11. Medium supplemented with oligosaccharides or plant growth-promoting rhizobacteria are alternative strategies to use with recalcitrant clones (see Notes 11–13).

### **3.7. Acclimatization to Ex Vitro Conditions**

1. Remove each rooted shoot carefully from the culture medium and rinse off the agar by washing roots with tap water without injuring (see Fig. 1h).
2. Transfer plants individually into 8 cm diameter plastic pots, each containing the acclimatization mixture (see *Potting media in Materials*) (see Fig. 1i) and well irrigate them.
3. Cover all potted plants with transparent plastic bags and grow them under acclimatization conditions (see Fig. 1i).
4. After 7 days, cut off a corner from the top of each bag to reduce the relative humidity and facilitate slow acclimatization of the plants to ex vitro conditions.
5. Gradually remove the remaining top corner of the bags after a further 2 weeks and discard the bags after an additional week (see Fig. 1j). (see Note 14).
6. Transfer plants to 12 cm diameter plastic pots in the glasshouse.
7. After 4–6 weeks in the glasshouse, plants develop their characteristic ex vitro aspect (see Fig. 1k). Plants showed stems with

opposite leaves, buds, and branches. Secondary thickening was observed in main shoot. Expanded elliptic leaves have thick wax layer and gray-green in color. Roots were well developed.

8. Suggested parameters for weekly evaluation: survival, number of nodes and shoot length per plantlet, rates of proliferation and elongation.

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## 4. Notes

1. The bacterial and mainly fungal contaminations are often major hurdle in establishing jojoba in vitro cultures and the losses reaching up to 90% (5). About 80% explants are successfully used for initiating in vitro cultures by using the disinfection protocol described here. Culture initiation from greenhouse-grown plant substantially reduces the contamination rates. Moreover, in vitro propagation by meristem culture is recommended for producing a large number of pathogen-free plants (18).
2. The explant collection time during the year is very crucial for the success of micropropagation. It is linked with the proliferation of microorganisms and the physiological condition of the plant which determines the degree of explant dormancy. In jojoba, the explants collected during spring show higher bud-break (15).
3. It is recommended that the protocol runs separately for each clone. Individual management of each clone permits the study of the in vitro responses of each genotype and reduces cross-contamination losses. Shoot proliferation, callus development, and hyperhydricity differ significantly among clones at all jojoba micropropagation stages (9–11, 13). Also, growth, flowering, wax production, and response to salinity are influenced by genotype (2, 3).
4. Cut leaf blades stimulate the sprouting of new shoots because the injury decreases leaf auxin synthesis, amending the local auxin/cytokinin ratio that stimulates the progression of buds from a transition stage to sustained bud outgrowth (19).
5. BA is more effective than kinetin (Kn) for inducing the growth of primary explants (9) (see Fig. 1a–d). Furthermore, nodal explants of different genotypes, as well as different sexes, may have differential requirements of cytokinins for shoot regeneration and medium-term conservation (11). In order to get the optimum jojoba proliferation in vitro, experiments with different proportions of sucrose and BA were conducted using the rotatable central composite design. This methodology uses

statistical and mathematical techniques for modeling and analyzing problems in which a response of interest is influenced by several variables. The best proliferation rate (5.7) is obtained in the presence of 2% sucrose and 7.2  $\mu\text{M}$  BA (20).

6. Hyperhydricity is a severe problem in *in vitro* culture. The term has been adopted to describe shoots with a vitreous and deformed appearance (see Fig. 2b). Some of the causes that lead to hyperhydricity are environmental factors, gelling agent properties, and growth regulators (21). The anatomy of normal *in vitro* leaves from micropropagated jojoba displays stomata at epidermal level, guard cells with a cuticular border and a thin cuticle with epicuticular waxes in the leaf epidermis (see Fig. 2a, c, d, g). *In vitro* hyperhydric shoots and leaves present numerous anatomical defects, e.g., short internodes, mesophyll, and stem cortex hypertrophy, malformed nonfunctional stomata some of which are occluded by a cuticular plug, epidermal discontinuity, and xylem hypolignification (see Fig. 2b, e, f, h, i). Explants with these features cannot survive to acclimatization (22).
7. Adventitious shoot formation in callus tissue can produce somaclonal variation, which can be used to generate some useful genetic variation in commercial cultivars. However it must be prevented to occur with utmost care especially for clonal propagation of elite germplasm/genotypes (18).
8. Ventilation during the proliferation stage affects the development of jojoba shoots. Variation in the response depends on the genotype, the extent of ventilation, and the shoot subculture number. In the case of some genotypes, the micropropagation protocol should include Magenta<sup>®</sup> boxes equipped with vented lids (or equivalent gas-permeable containers) as growing vessels to improve growth and reduce hyperhydricity (23).
9. Pool jojoba clones cultured in medium supplemented with auxin during 45 days, showed the best rooting with 14.8  $\mu\text{M}$  IBA (33% rooted plants). However, when is used only 7 days pulse IBA treatment (14.8  $\mu\text{M}$ ), 50% of plants rooted (9, 12).
10. Root surface area from *in vitro* plants can be determined by immersing air-dried roots in  $\text{Ca}(\text{NO}_3)_2$  saturated solution and determine salt quantity (mg), removed from the solution, which is sticking to the root surface (24).
11. Trehalose, a nonreducing disaccharide, plays an important physiological role as a storage carbohydrate and protector from abiotic stresses. In the initiation stage, explants cultured in the hormone-free medium containing 1 mM trehalose show a significant increase in the proliferation rate (3.2) when compared with phytohormone control medium (2.4) (Fig. 3a). In the proliferation stage, a shoot proliferation

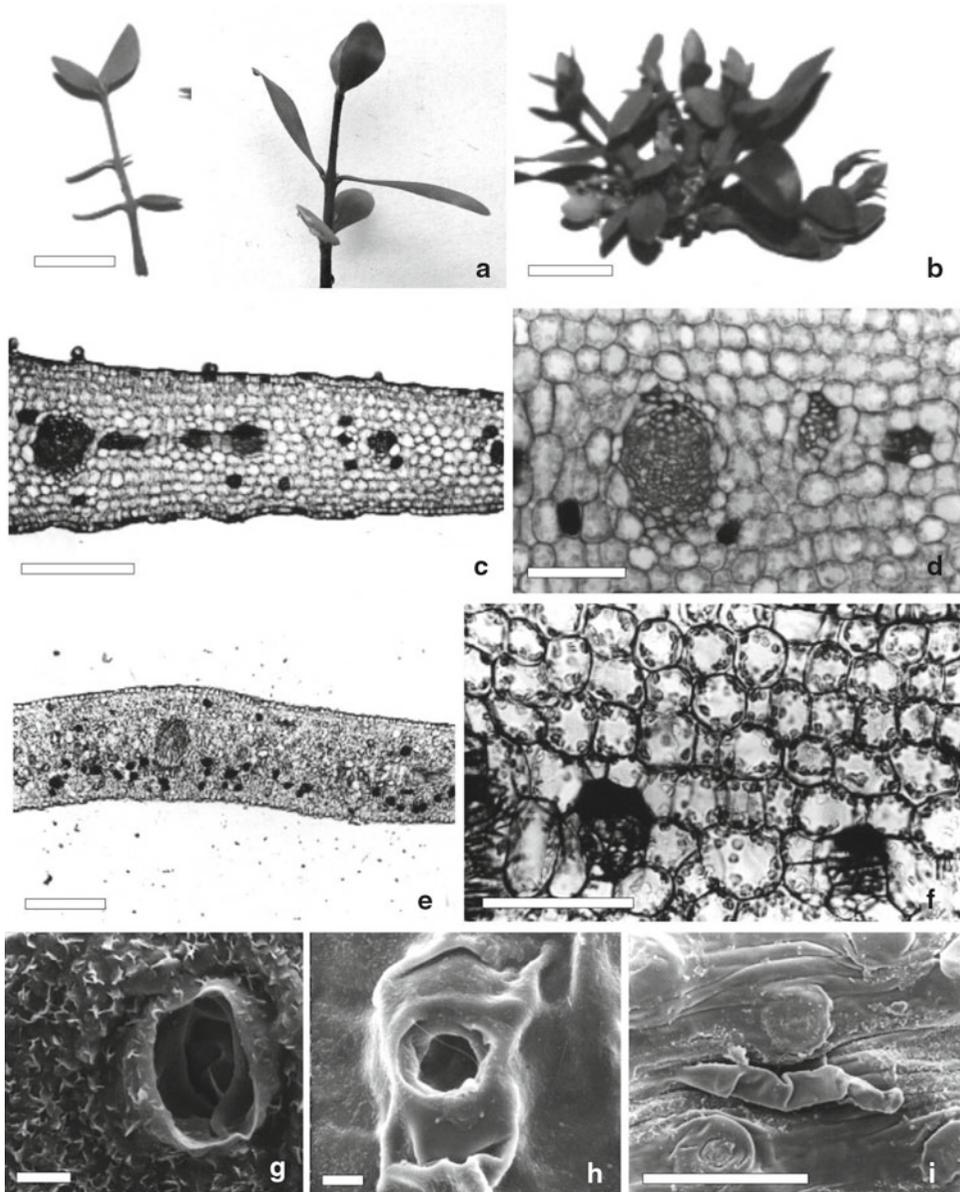


Fig. 2. Morphology and anatomy of in vitro jojoba in shoot proliferation. (a) Normal shoots; (b) Hyperhydic shoots; (c, d) Transverse section of normal leaves (LM); (e, f) Transverse section of hyperhydic leaves (LM); (g) Normal leaf surface, stomata (SEM); (h, i) Hyperhydic leaf surface (SEM). LM light microscopy; SEM scanning electron microscopy. Bars: a, b: 1 cm; c, e: 250  $\mu$ m; d, f: 50  $\mu$ m; g, h: 10  $\mu$ m; i: 100  $\mu$ m. (Figure 2g–i, reproduced from Llorente et al. (12) and Apóstolo and Llorente (22), with permission from Springer Science + Business Media BV).

rate of 4.8 is achieved in BM with 4.44  $\mu$ M BA and 1 mM trehalose. Also, the addition of trehalose to the IBA-rhizogenesis media decreases basal callus (12).

12. Addition of cyclodextrins to modified MS (16) culture medium containing IBA as 1 week pulse treatment doubles rooting

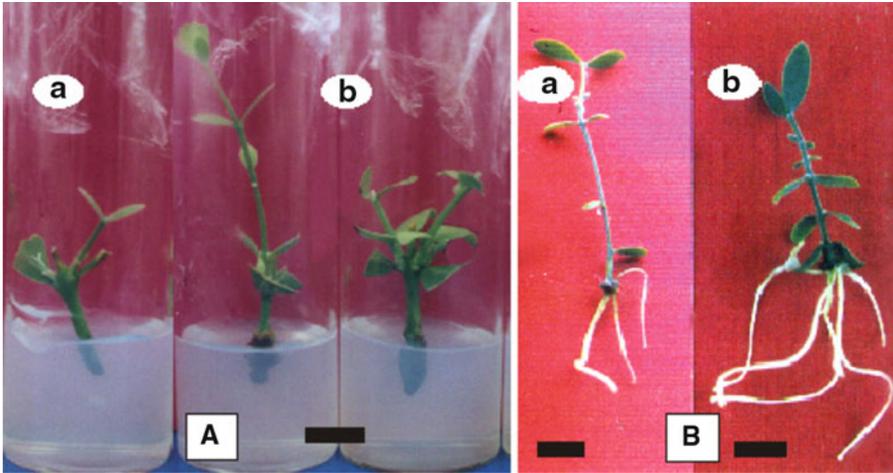


Fig. 3. Effect of oligosaccharides on jojoba micropropagation. (A) Trehalose effect on initiation cultures: (a) shoots in basal medium (BM) supplemented with 0.49  $\mu\text{M}$  IBA, 1.44  $\mu\text{M}$   $\text{GA}_3$  and 11.10  $\mu\text{M}$  BA; (b) shoots in BM supplemented with trehalose 1 mM. (B)  $\beta$ -Cyclodextrins ( $\beta$ -Cd) effect on in vitro rooting: (a) shoot rooting in rooting medium (RM) supplemented with 15  $\mu\text{M}$  IBA (control treatment); (b) shoot rooting in RM supplemented with 15  $\mu\text{M}$  IBA and 30  $\mu\text{M}$   $\beta$ -Cd. Bars: a, b: 1 cm (Fig. 3 Ba, 3 Bb reproduced from ref. (25) with permission from Springer Science + Business Media BV).

percentage and induces earlier rooting with respect to IBA control medium (see Fig. 3b). Increase in rooting percentage after treatment with cyclodextrins was also observed in the absence of IBA, indicating that both compounds promote rooting per se (25).

13. A plant growth-promoting rhizobacterium (*Azospirillum brasilense* strain Cd) stimulates in vitro rooting of jojoba. Explants grown for 4 days on a medium supplemented with 14.8  $\mu\text{M}$  IBA were transferred to hormone-free medium and inoculated at with 0.1 mL of bacterial culture at the base of each explant ( $2 \times 10^7$  colony forming units). The inoculated explant reached a rooting percentage of 86% and rooted 5 days earlier than the controls. Also, they showed a significant increase in the mean number of roots per shoot, and exhibited less callus production than the controls (19).
14. The transition from in vitro to ex vitro conditions can be difficult. Shoots growing in vitro have been continuously exposed to a microenvironment under low light intensity, aseptic conditions, and high humidity. The micropropagated plants undergo some anatomical and physiological changes. High humidity in the in vitro culture conditions should be gradually reduced to allow plantlets to develop normal cuticle and stomata and to avoid dehydration during acclimatization (26). Likewise, relatively low levels of light produce thin leaves and it is necessary to put them under the shade for 4 weeks and gradually move them to the ex vitro light level, especially during summer months (27).

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## In Vitro Propagation of Olive (*Olea europaea* L.) by Nodal Segmentation of Elongated Shoots

Maurizio Lambardi, Elif Aylin Ozudogru, and Romano Roncasaglia

### Abstract

Olive (*Olea europaea* L.), long-living, ever-green fruit tree of the Old World, has been part of a traditional landscape in the Mediterranean area for centuries. Both the fruits consumed after processing and the oil extracted from the fruits are among the main components of the Mediterranean diet, widely used for salads and cooking, as well as for preserving other food. Documentations show that the ancient use of this beautiful tree also includes lamp fuel production, wool treatment, soap production, medicine, and cosmetics. However, unlike the majority of the fruit species, olive propagation is still a laborious practice. As regards traditional propagation, rooting of cuttings and grafting stem segments onto rootstocks are possible, former being achieved only when the cuttings are collected in specific periods (spring or beginning of autumn), and latter only when skilled grafters are available. In both the cases, performance of the cultivars varies considerably. The regeneration of whole plants from ovules, on the other hand, is used only occasionally. Micropropagation of olive is not easy mainly due to explant oxidation, difficulties in explant disinfection, and labor-oriented establishment of in vitro shoot cultures. However today, the progress in micropropagation technology has made available the complete protocols for several Mediterranean cultivars. This chapter describes a micropropagation protocol based on the segmentation of nodal segments obtained from elongated shoots.

**Key words:** Axillary buds, Micropropagation, *Olea europaea* L., Olive, Zeatin

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### 1. Introduction

Olive (*Olea europaea* L.), belonging to the genus *Olea* of the Oleaceae family, is one of the most ancient domesticated fruit trees of the Old World (1). It is traditionally grown in the Mediterranean area, and countries bordering the sea include 95% of the global olive orchards, with a production of about 97% olive oil and 75% table olives. The main producers are Spain, Italy, and Greece for olive oil, and Spain and Turkey for table olives (2, 3).

The first signs of olive cultivation in the eastern Mediterranean coasts and islands, covering today's eastern Turkey, western Iran, Lebanon, northern Israel, Syria, and northern Iraq, date back to 4th millennium bc (1, 4, 5). Since then it moved westwards to Greece and the Aegean archipelago, although probably Crete and Cyprus belonged to the oldest olive growing center. In the beginning of the first millennium bc, olive introduction continued to spread westwards, to Sicily and Tunisia. From there, the crop was introduced by the Romans to many other places in the Mediterranean area, including Spain, France, and northern Africa. With the fall of the Roman empire olive cultivation dropped dramatically due to the reduction in population and abandonment of cultivated areas, however it regained its importance in Europe during sixteenth and seventeenth centuries. Production and distribution of olive outside the traditional cultivated areas continues to expand, recently reaching USA, Australia, China, South Africa, Japan, New Zealand, India, and Pakistan (2, 3).

Olive is a slow-growing, long-living, evergreen, medium-sized (4–8 m high) fruit tree with a characteristic of a basitonous growth habit. The trunk has a smooth surface when young, however tends to get rough and twisted as grows further. Unpruned canopy forms a dense globe. The slender shoots carry either flower or fruit buds, while mixed buds appear rarely (3). As a Mediterranean tree, it can easily adapt to difficult soil conditions and very high temperatures; however, it performs best in temperate dry climates where sharp and prolonged temperature falls are not seen. The tree has a long juvenile period, starting to bear fruits 3–4 years after planting; however, if managed properly it continues fruiting for hundreds of years (6).

Oil extracted from the fruits is an important product of olive cultivation. It is used for salads and cooking, as well as for preserving other foods. In ancient times, olive oil was also used as lamp fuel, wool treatment, medicine, cosmetics, and soap production (3). Table olives are also typical components of the Mediterranean diet. In the Mediterranean countries, the olive has been closely associated with the traditional landscape, as well.

There are several traditional propagation techniques available for olive propagation, including rooting of leafy stem or softwood cuttings, grafting stem segments onto seedlings or rootstocks, and plant regeneration from ovules, although the latter is used only occasionally (2). Among all the available techniques, rooting of the leafy stems is widely used since the mid-1950s. However, still several problems are encountered while using this technique. For instance, (i) rooting of the cuttings is successful only when cuttings are collected in spring (before blossoming) or at the onset of autumn growth, (ii) adventitious rooting capacity varies among cultivars, (iii) different cultivars perform very differently, even

when similar parameters such as physiological, agronomical, and propagation conditions are applied. As for grafting propagation, due to the high costs and lack of selected rootstocks, the technique is only partially used in some countries, such as Italy, being laborious and requiring skilled grafters (7). Thereby, micropropagation is an ideal tool for clonal propagation of selected olive cultivars or clones (6). However, olive micropropagation is not easy practice due to some problems, e.g., oxidation of explants, difficulties in disinfection, laborious in establishing shoot cultures, and it is highly cultivar dependent. In the beginning of the 1990s, only few olive cultivars could be efficiently propagated *in vitro* by axillary bud development and shoot proliferation (3, 6, 8–10). Thanks to the advances in the technology and optimizations of various steps of olive micropropagation, complete protocols are today available to propagate several cultivars from different Mediterranean countries (6, 11, 12). In this chapter, the micropropagation protocol based on the elongation of uni- and binodal segments on semisolid medium and establishment of shoot cultures by their re-segmentation is described.

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## 2. Materials

### 2.1. Medium Preparation

1. Deionized high quality water.
2. Stock solutions for inorganic salts and organic elements of Olive Medium (OM (13)) (see Notes 1 and 2).
3. Stock solutions for zeatin, gibberellic acid (GA<sub>3</sub>), indole-3-butyric acid (IBA), and 1-naphthaleneacetic acid (NAA).
4. Mannitol.
5. Agar or gelrite.
6. Beakers (100, 1,000 mL).
7. Pipette or graduated cylinders (100, 1,000 mL).
8. Magnetic stir bars.
9. 0.22 μM syringe filters.
10. Syringes.
11. 1 M HCl and NaOH.
12. 500 mL glass jars.
13. Test tubes.
14. Plastic film.
15. Tissue culture facilities: balance, analytical balance, magnetic stirrer, pH meter, microwave oven or hot plate, refrigerators at 4 and -20 °C, autoclave.

**2.2. Disinfection of the Explants**

1. Running tap water.
2. Disinfection solution containing 11% NaOCl and 0.035% HgCl<sub>2</sub> (9).
3. Sterile dH<sub>2</sub>O.
4. Sterile beakers (100 mL).
5. Proliferation medium contained in test tubes.
6. Tissue culture facilities: vacuum, laminar air flow cabinet, glass bead sterilizer or spirit burner, stainless steel scalpels and forceps, climatic chamber.

**2.3. Shoot Proliferation and Rooting**

1. OM medium contained in 500 mL glass jars (added of zeatin for shoot proliferation, GA<sub>3</sub> for shoot elongation, and IBA or NAA for shoot rooting).
2. Plastic film.
3. Sterile Petri dishes (90 mm Ø).
4. Tissue culture facilities: laminar air flow cabinet, glass bead sterilizer or spirit burner, stainless steel scalpels and forceps, climatic chamber.

**2.4. Plantlet Acclimatization**

1. Lukewarm water.
2. Plastic or paper containers.
3. Free-draining compost substrates (peat moss, perlite, polystyrene granules, 2:2:1).
4. Fungicide.
5. Tap water.
6. Tunnel covered with transparent plastic film.

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**3. Methods****3.1. Preparation and Sterilization of the Culture Media**

1. Add deionized high-quality water (one-half of the final volume) into a large beaker.
2. Stock solutions of inorganic salts and organic elements are prepared according to the formulations given in Table 1. While stirring, add required amounts of each ingredient from stock solutions by pipette or graduated cylinder (see Note 3).
3. Add appropriate amounts of mannitol and agar (or gelrite).
4. If elongation or rooting medium is prepared, GA<sub>3</sub>, IBA, or NAA can be added at this time.
5. Add water to reach the prescribed final volume.

**Table 1**  
**OM medium formulation (13), used for the multiplication and rooting of the olive shoots**

Stock solutions	Preparation of the Stock Solutions (mg/L)	Preparation of OM Media (ml/L) <sup>a</sup>
Macroelement stocks		
KNO <sub>3</sub>	1,100	10.9
NH <sub>4</sub> NO <sub>3</sub>	412	5.1
CaCl <sub>2</sub> × 2H <sub>2</sub> O	440	12.0
MgSO <sub>4</sub> × 7H <sub>2</sub> O	1,500	24.3
KH <sub>2</sub> PO <sub>4</sub>	340	10.0
Ca(NO <sub>3</sub> ) × 4H <sub>2</sub> O	600	10.2
KCl	500	
FeSO <sub>4</sub> × 7H <sub>2</sub> O + Na <sub>2</sub> EDTA	27.8 + 37.5	20.0
Microelement stock (MS)		10
MnSO <sub>4</sub> × 4H <sub>2</sub> O	22.3	
Na <sub>2</sub> MoO <sub>4</sub> × 2H <sub>2</sub> O	0.25	
CoCl <sub>2</sub> × 6H <sub>2</sub> O	0.025	
KI	0.83	
Microelement stock (OM)		10
H <sub>3</sub> BO <sub>3</sub>	12.4	
ZnSO <sub>4</sub> × 7H <sub>2</sub> O	14.3	
CuSO <sub>4</sub> × 5H <sub>2</sub> O	0.25	
OM Vitamin		10
Glycine	2.0	
Thiamine HCl	0.5	
Pyridoxine HCl	0.5	
Nicotinic acid	5.0	
Biotine	0.05	
Folic acid	0.5	
Myo-inositol <sup>b</sup>		100 mg/L
Glutamine		2,194 mg/L
Mannitol		36 g/L
Agar (or Gelrite)		5–8 g/L (or 2–3 g/L)
Zeatin (for proliferation)		1–4 mg/L
GA <sub>3</sub> (for elongation)		20–40 mg/L
IBA (for rooting)		2–3 mg/L
NAA (for rooting)		1 mg/L

<sup>a</sup>Note that for macroelements, stock solutions are prepared separately, while for microelements and organic elements unique solutions containing a mixture are prepared

<sup>b</sup>These quantities are added directly to the 1 L medium

6. Adjust the pH of the solution to 5.7–5.8 by adding drop by drop 1 M HCl or NaOH.
7. Heat the solution (in a microwave oven or on a hot plate) to melt the agar.
8. When the agar is melted completely, dispense the solution into test tubes and glass jars (used for in vitro introduction of explant and shoot proliferation, respectively), which are then closed with a plastic film and autoclaved at 121 °C for 20 min.
9. After the autoclavage, cool the jars and tubes to below 70 °C before being taken out of the autoclave.
10. For proliferation medium, when the medium is cooled to about 50 °C, add previously filter-sterilized zeatin under laminar flow hood and shake gently (see Note 4). To prepare 100 mL stock solution of 200 mg/L concentration, dissolve 20 mg zeatin with few drops of 1 M NaOH in 100 mL beaker. Stir while adding distilled water. Transfer into a 100 mL graduated flask and bring the final volume to 100 mL. To have a medium with 1 mg/L zeatin concentration, add 5 mL stock solution to 1 L culture medium (see Note 5).
11. The medium is ready for use when gelifies and reaches room temperature.

### **3.2. Collection and Disinfection of Explants**

The best material to initiate in vitro propagation of olive is the potted stock plants maintained under greenhouse conditions (see Fig. 1a). Those can be established by grafting scions of elite cultivars, selected for superior phenotypic characteristics, genetic stability, and good sanitary conditions, onto potted seedlings (see Notes 6 and 7). Alternatively, olive stock plants can be established by cutting propagation (2).

In olive, the best explants to initiate in vitro cultures are tender apical twigs and uninodal segments (see Fig. 1b), excised from vigorous shoots soon after spring sprouting. Occasionally, bi- or trinodal segments are used (9).

1. Rinse the explants in running tap water for at least 30 min (see Note 8).
2. Treat with a solution containing 11% NaOCl and 0.035% HgCl<sub>2</sub> for 3–5 min under vacuum (9).
3. Rinse in sterile distilled water for 30–40 min.
4. Culture the explants in test tubes for 4–6 weeks in order to avoid the diffusion of contamination (see Fig. 1c).

### **3.3. In Vitro Shoot Proliferation and Rooting of the Shoots**

Olive shoots are characterized by a strong apical dominance, thus the proliferation of shoots through the stimulation of axillary buds is of minor importance. Instead, shoot multiplication is achieved



Fig. 1. In vitro propagation of olive. (a) Potted stock plants, maintained under greenhouse conditions, serve as explant source, (b) uninodal segments used for culture initiation, (c) elongated shoots ready for subculturing. Note that cultures are initiated in test tubes to avoid the spread of contamination, (d) in vitro growth habit of different olive cultivars. Note that cvs. Arbequina (*left, top*) and Canino (*left, bottom*) proliferate in the mode of elongation, while Ascolana (*right, top*) and Coratina (*right, bottom*) also produce multiple shoots, (e) typical subculture of elongated shoots by uni- or binodal segmentation, (f) appearance of glass jars at the end (*left*) and at the beginning (*right*) of the subculture period, (g) rooted plantlets ready for acclimatization, (h) olive plants after 2 months of acclimatization (in tunnel), followed by 2 months of hardening (outside tunnel). (Photos **b**, **c** and **f** are reproduced from Olive Propagation Manual (2)).

by means of uni- or binodal segmentation of elongated shoots at each subculture (2, 6).

1. Following the first transfer in test tubes, 2–3 cm long micro-shoots are formed from disinfected cultures. Transfer uni- or binodal segments on 100–150 mL of semisolid OM medium supplemented with 1–4 mg/L zeatin (see Note 9), 30–36 g/L mannitol (see Note 10), and 5–8 g/L agar (or 2–3 g/L gelrite), contained in 500 mL glass jars (15–20 segment per jar) (see Note 11), closed with glass lid and wrapped in plastic film (see Note 12).
2. Maintain the cultures in climatic chamber, at 23–24 °C, 16 h photoperiod, and 40–60  $\mu\text{mol}/\text{m}^2/\text{s}$  of photosynthetically active radiation provided by cool-white fluorescent lamps.
3. Dissect uni- or binodal segments (2–3 cm long) of elongated shoots and subculture on fresh medium at every 4–7 week interval, depending on the cultivar, to maintain the prolific growing shoot cultures (see Fig. 1d–f).
4. Some cultivars require a shoot elongation period before rooting (see Note 13). For this, transfer the plantlets to fresh medium containing 20–40 mg/L  $\text{GA}_3$  according to the elongation capacity of the specific cultivar.
5. For rooting, after 2–3 weeks, when shoots are elongated to 3–4 cm, transfer them on  $\frac{1}{2}$  strength OM medium, containing 2–3 mg/L IBA or 1 mg/L 1-NAA, according to the rooting capacity of the specific cultivar (see Notes 14 and 15).

### **3.4. Acclimatization of the Plantlets**

1. Remove the rooted plantlets (see Fig. 1g) from the glass jars and wash gently roots in luke warm water to remove agar sticking to roots (see Note 16).
2. Insert the plantlets into plastic or paper containers, containing appropriate free-draining compost substrates (e.g., peat moss, perlite, and polystyrene granules, 2:2:1 (9)) (see Note 17).
3. Transfer them to a tunnel covered with transparent plastic film or in mist or fog conditions (see Note 18).
4. As soon as they are set out, give fungicide treatment, e.g., 60 g/L SWITCH™ (Syngenta) containing 37.5% Cyprodinil and 25% Fludioxonil, by spraying plantlets and substrate.
5. Irrigate plants periodically to maintain the appropriate level of relative humidity (RH, 85–90%) (see Note 19).
6. Gradually reduce the RH by opening the tunnel for increasing periods of time or by reducing the frequency of fogging/misting.
7. Following acclimatization, plants require 1-year hardening period before planting in the field (see Note 20, Fig. 1h).

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## 4. Notes

1. A specific medium (as regards inorganic salts and organic elements) is available for olive, formulated on mineral element analysis of shoot apices from field plants during their rapid growth (13). The medium known as Olive Medium (OM) is richer than the widely used MS (Murashige and Skoog) medium (14) in Ca, Mg, S, P, B, Cu, and Zn; it has a slightly different Ca/N ratio (1:11) and contains glutamine as a nitrogen source (15).
2. Stock solutions of inorganic salts are stored at 4 °C, while stock solutions of organic elements are stored at -20 °C.
3. Alternatively, an appropriate amount of the commercial OM preparation (Duchefa, The Netherlands), containing microelements, macroelements, and vitamins, can be used.
4. All cytokinins are unstable during autoclaving. Zeatin, in particular, can lose large part of its efficiency during sterilization at 121 °C. Therefore, it is suggested to filter-sterilize stock solution and add appropriate amount directly to the culture jars after autoclave.
5. Similar procedures can be followed to prepare stock solutions of GA<sub>3</sub> and auxins added to the medium for shoot elongation and rooting of the shoots (see Subheading 3.3, step 5).
6. If an old (or a senescent) tree has to be reproduced, stock plants very reactive to propagation *in vitro* can be achieved through two or more cycles of repeated graftage (“serial graftage,” (16)). The technique produces strong effect of rejuvenation of the propagation material.
7. From explants taken from adult field trees, initiation of *in vitro* cultures is difficult and time consuming, mainly due to rapid oxidation of the tissues. This can be minimized by using juvenile vegetative structures such as vigorous upgrowing shoots, suckers, and ovules as an explant source (17). Alternatively, culture medium can be supplemented with antioxidants, such as ascorbic acid (10–20 mg/L) or citric acid (100–200 mg/L), or explants can be dipped in a sterile antioxidant solution before *in vitro* culture (2).
8. Avoid the use of ethanol to disinfect explant for the prevention of tissue dehydration (6).
9. Zeatin induces highest stimulation of olive axillary and apical buds. Concentration varies from 0.5 to 10 mg/L depending on the genotype; however, 1–4 mg/L concentration range is the most suitable for shoot proliferation and/or elongation in majority of the olive cultivars (6). A lower proliferative efficiency is reported when zeatin is replaced by other cytokinins. On the

contrary, a combination of zeatin with 6- $\gamma,\gamma$ -dimethylallylamino-purine (2iP) and thidiazuron (TDZ) seems promising, although restricted to one or few cultivars (18).

10. In olive, mannitol increases shoot proliferation, improves the quality and the uniformity of shoot cultures, and reduces basal callus formation (19).
11. Glass jar is the primary choice of the commercial micropropagation laboratories. Alternatively, polypropylene containers, such as ECO2 Box and Steri Vent (Duchefa, The Netherlands, 650 mL) can be used, having closures that allow continuous gas exchange and thus reduce accumulation of volatile compounds, such as ethylene and CO<sub>2</sub> (2).
12. In alternative to the use of semisolid medium, a semiautomatic temporary immersion systems for high rate *in vitro* proliferation of olive binodal segments can be used (12). In this procedure, the shoots are cultured in a RITA<sup>®</sup> cylindric container (VITROPIC, France), containing 200 mL liquid OM proliferation medium, which are connected to a timer to adjust 16 min immersion of the shoots in the liquid medium in every 16 h by pneumatic transfers, providing 24 min daily immersion, ventilation of the culture vessels, and intermittent contact of the explants with the medium. The method is proved to be effective in producing higher proliferation rate, increased length of internodes, lower density of stomata, greater quantity of wax, and trichomes in the leaf surface.
13. For cultivars producing 3–4 cm long shoots during proliferation stage, the elongation step can be omitted.
14. Alternatively, Bartolini et al. (20) proposed a “dip rooting method,” where microshoots are dipped into a concentrated solution of potassium salt-IBA for 30 min before transfer on hormone-free rooting medium. Similarly, Rugini and Fedeli (9) succeeded in inducing high rooting frequency rate by dipping the entire microshoots or their basal parts into an IBA solution (100–200 mg/L) for 10–20 s.
15. Olive rooting is also promoted by basal etiolation of the shoots by painting the jars with black paint; covering the rooting medium with black, sterile, polycarbonate granules; and addition of 1 mM putrescine to an auxin-containing rooting medium (21, 22).
16. Remove carefully agar sticking to roots of plantlets in order to avoid plantlet contamination, which is caused by sucrose and other organics present in agar medium. Soak roots in a fungicide (e.g., Switch<sup>™</sup>, see subheading 3.4, step 4) in combination, when necessary, with an anti-bacterial solution (e.g., D50 containing 5% stabilized peracetic acid, 20% hydrogen peroxide, with a combination of surfactants, buffering agents, and stabilizing agents, at a 5ml/L concentration).

17. The compost substrate should be sanitized to eliminate bacterial or fungal infections.
18. Plastic tunnels are the cheapest system for the acclimatization of olive plants. However, they can become overheated during the hot summer periods, and may need shading, e.g., with a 50% shade cloth. Temperature and humidity should be monitored periodically.
19. For preventing the promotion of algal growth, and fungal and bacterial attacks to the plants, avoid flooding of compost substrate during acclimatization.
20. Micropropagated olive trees do not exhibit prolonged juvenile traits under the field conditions and bear fruits in the third or fourth growing season.

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# Chapter 4

## Micropropagation Systems of Feijoa (*Acca sellowiana* (O. Berg) Burret)

Miguel Pedro Guerra, Gabriela Claudia Cangahuala-Inocente,  
Lirio Luiz Dal Vesco, Rosete Pescador, and Clarissa Alves Caprestano

### Abstract

*Acca sellowiana* (O. Berg) Burret sin. *Feijoa sellowiana* (Myrtaceae) is a semiwoody fruit species native to South Brazil, Uruguay, and Argentina; edible fruits are tasty. The naturally occurring populations in Santa Catarina State show high variability in fruit size, color, and other features. A breeding program launched in 1990 resulted in the release of four Brazilian commercial varieties. The conventional clonal propagation methods of this species, such as cutting and grafting, have shown low efficiency. Therefore, tissue culture techniques were developed for mass propagation. This chapter describes several protocols based on organogenesis and somatic embryogenesis. Additional techniques including synthetic seed technology and temporary immersion system are also described.

**Key words:** Artificial seed, *Feijoa sellowiana*, Mass propagation, Myrtaceae, Organogenesis, Somatic embryogenesis, Tissue culture, Zygotic embryo

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## 1. Introduction

*Acca sellowiana* (O. Berg) Burret sin. *Feijoa sellowiana* (Myrtaceae) is native to the southern region of Brazilian Plateau and also found in Uruguay and Argentina (1). It naturally occurs in the highlands of the Atlantic Forest Biome and shows high genetic variability (2, 3) and produces tasty fruits (4) called “Goiaba-Serrana” in Brazil, and Feijoa and Pineapple Guava in other parts of the world. Although this plant has been cultivated commercially in New Zealand, Australia, USA, and some European countries since the beginning of the last century, it has recently been domesticated and commercially cultivated in Brazil (5, 6). The conventional methods of clonal propagation, such as cutting and grafting, are inefficient (7). Considering this, tissue culture techniques were

developed for mass propagation of *A. sellowiana* by organogenesis (8). Among salt formulations woody plant medium (WPM) was the most responsive (9). It was also shown that the basal culture medium devoid of plant growth regulators (PGR) was effective in the induction of organogenesis (10). The production of adventitious shoots from young leaves was also reported (11). Culturing of nodal segments and apical meristems showed low rates of bud neoformation (12). Nodal segments of the genotype 101 showed high regeneration rates on WPM medium devoid of PGR. Ex vitro rooting was obtained with 100 mM indole-3-butyric acid (IBA) treatment for 60 min (13).

Since the induction of somatic embryogenesis in these species, protocols have been improved (14). The number of somatic embryos has increased on liquid plant medium (LPm, see ref. (15)) containing 4 mM glutamine (Gln) (16). The half strength Murashige and Skoog (MS, see ref. (17)) culture medium supplemented with 0.5  $\mu$ M benzylaminopurine (BAP) ideally germinated somatic embryos into plantlets. However, somatic embryogenesis was genotypic dependent (18), and 2,4-Dichlorophenoxyacetic acid (2,4-D) pulse treatment induced somatic embryogenesis. It has been postulated that genetic reprogramming of explant tissues culminating with the induction of somatic embryogenesis is associated with the methylation of nuclear DNA modulated by the 2,4-D supplemented to the culture medium (19). Cangahuala-Inocente et al. (20) showed high rates of embryogenetic induction (100%) and number of somatic embryos/explant in response to glutamic acid (Glu) and 2,4-D. The quality of somatic embryos was improved with abscisic acid (ABA) (21). Filament of stamens inoculated in LPm supplemented with several types and levels of PGR resulted in the development of somatic embryos (22).

A schematic representation of the modulation of somatic embryogenesis in *A. sellowiana* is presented in Fig. 1. The differences among the protocols are mainly attributed to the genotype-dependent response of *A. sellowiana* to tissue culture techniques.

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## 2. Material

### 2.1. Micropropagation by Organogenesis

#### 2.1.1. Surface Sterilization of Source Material

1. Potted plants as a source of explants.
2. Benlate®.
3. Ethanol 70% (v/v).
4. Sodium hypochlorite (NaOCl) with 5% of active Cl.
5. Tween 20.
6. Sterile distilled water.
7. Ascorbic acid.
8. Citric acid.

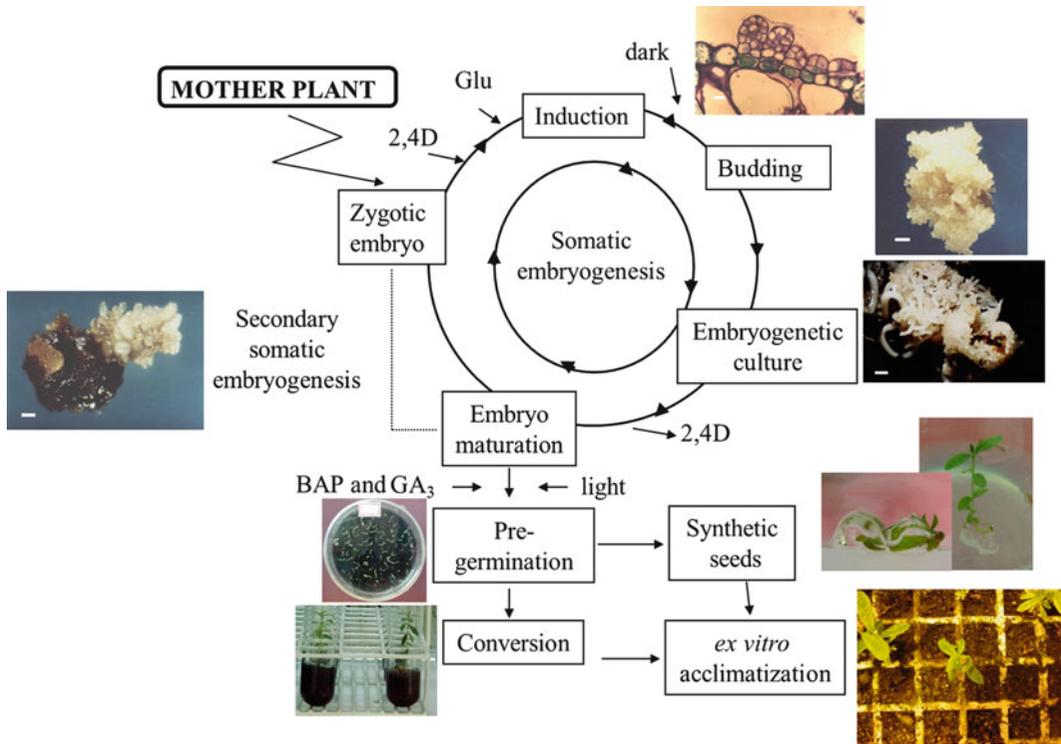


Fig. 1. Modulation of somatic embryogenesis in *Acca sellowiana*.

9. Sterile Whatman® No. 1 filter paper.
10. Mercury (II) chloride ( $\text{HgCl}_2$ ).

#### 2.1.2. Culture Media Composition

1. Media based on Murashige and Skoog—MS salts (17). See media formulations in Table 1.
2. Media based on WPM salts (9) (see Table 1).
3. Morel's vitamins (23) (see Table 1).
4. Staba vitamins (24) (see Table 1).
5. Sucrose.
6. Activated charcoal.
7. PGR BAP, IBA, 1-Naphtaleneacetic acid (NAA), Gibberellic acid ( $\text{GA}_3$ ), 6-( $\gamma,\gamma$ -Dimethylallylamino) purine (2-iP), N6-furfuryladenine (Kinetin), and 4-Amino-3,5,6-trichloro-2-pyridinecarboxylic acid (Picloram).
8. Difco Agar.

#### 2.1.3. General Supplements

1. Tissue culture facilities—instruments (scalpel, forceps, spirit burner to flame sterilize instruments), laminar flow cabinet, culture room, pH meter, Phytotron autoclave, test tubes, bottles.
2. Sodium hydroxide (NaOH) (0.5 N).
3. Hydrochloric acid (HCl) (0.5 N).

**Table 1**  
**Saline and vitamin formulations of basal culture media**

		MS (17)	WPM (9)	LPm (15)
		mg/L	mg/L	mg/L
<i>Macronutrients</i>				
Ammonium nitrate	NH <sub>4</sub> NO <sub>3</sub>	1650	400	1200
Potassium nitrate	KNO <sub>3</sub>	1900	–	1900
Calcium chloride	CaCl <sub>2</sub> × 2H <sub>2</sub> O	440	96	180
Calcium nitrate	Ca(NO <sub>3</sub> ) <sub>2</sub>	–	556	–
Potassium phosphate	KH <sub>2</sub> PO <sub>4</sub>	170	170	340
Potassium sulfate	K <sub>2</sub> SO <sub>4</sub>	–	990	–
Magnesium sulfate	MgSO <sub>4</sub> × 7H <sub>2</sub> O	370	370	370
<i>FeEDTA solution</i>				
Sodium EDTA	Na <sub>2</sub> EDTA × 2H <sub>2</sub> O	37.2	37.3	19.0
Ferrous sulfate	FeSO <sub>4</sub> × 7H <sub>2</sub> O	27.8	27.8	14.0
<i>Micronutrients</i>				
Zinc sulfate	ZnSO <sub>4</sub> × 7H <sub>2</sub> O	8.6	8.6	2.88
Manganese sulfate	MnSO <sub>4</sub> × H <sub>2</sub> O	16.9	16.9	1.69
Potassium iodide	KI	0.83	–	0.75
Boric acid	H <sub>3</sub> BO <sub>3</sub>	6.2	6.2	0.63
Sodium molybdate	Na <sub>2</sub> MoO <sub>4</sub> × 2H <sub>2</sub> O	0.25	0.25	0.025
Cupric sulfate	CuSO <sub>4</sub> × 5H <sub>2</sub> O	0.025	0.25	0.0025
Cobalt chloride	CoCl <sub>2</sub> × 6H <sub>2</sub> O	0.025	–	0.0025
<i>Vitamins</i>				
	Morel (23) mg/L		Staba (24) mg/L	
Myo-inositol	100		2000	
Thiamine HCl	1.0		1.0	
Nicotinic acid	1.0		2.0	
Pyridoxine HCl	1.0		2.0	
Pantothenate	1.0		1.0	
D-Biotin	0.01		1.0	
Choline chloride	–		1.0	
4-aminobenzoic acid	–		0.5	
Folic acid	–		0.5	

Add sucrose (30 g/L). Adjust the pH of the medium to 5.8 with 0.5 N NaOH, autoclave at 121 °C for 16 min

#### 2.1.4. Ex Vitro Rooting

1. Trays containing 72 cells (60 cm<sup>3</sup> each).
2. Substrate: mixed commercial substrate of vermiculite and carbonized rice coat (1:1; v/v).
3. Plastic box covered with glass.

#### 2.2. Micropropagation by Somatic Embryogenesis

##### 2.2.1. Explant Sterilization

1. Immature seeds.
2. Mature seeds.
3. Floral buds.
4. Ethanol 70% (v/v).

5. Sodium hypochlorite (NaClO) with 5% of active Cl.
6. Sterile distilled water.

### *2.2.2. Culture Media Composition*

1. Media based on L<sub>P</sub>m salts (15). See media formulations in Table 1.
2. Morel's vitamins.
3. Sucrose and maltose
4. Phytigel®.
5. Difco Agar.
6. Glutamine (gln).
7. Myo-inositol.
8. Glutamic acid (glu).
9. Plant growth regulators (BAP, GA<sub>3</sub>, 2,4-D, 2-ip, Picloram, kinetin).
10. Fluridone.

### *2.2.3. General Supplements*

1. Tissue culture facilities—instruments (scalpel, forceps, spirit burner to flame sterilize instruments), laminar flow cabinet, culture room, pH meter, Phytotron autoclave, test tubes, Petri dishes, nipple-flasks.
2. Stereomicroscope.
3. Celetor apparatus (Sigma).
4. Steward apparatus.
5. Carmine.
6. Acetic acid 45% (v/v).
7. Evan's Blue.
8. Reflux condenser.
9. Slides and coverslip.
10. Light microscope.
11. RITA® bioreactor.
12. Substrate PlantMax®.
13. Vermiculite.
14. Trays containing 72 cells (60 cm<sup>3</sup> each).
15. Plastic box covered with glass.

### **2.3. Synthetic Seeds**

1. Torpedo-staged somatic embryos.
2. Media based on L<sub>P</sub>m salts.
3. Morel vitamins.
4. Sucrose.
5. Difco Agar.
6. Activated charcoal.

7. Plant growth regulators (BAP, GA<sub>3</sub>).
8. Petri dishes (9 × 120 cm diameter).
9. Alginate Sodium Salt from brown algae (Fluka).
10. Calcium chloride (CaCl<sub>2</sub>).
11. Potassium nitrate (KNO<sub>3</sub>).
12. Distilled water.
13. Tissue culture facilities—instruments (scalpel, forceps, micropipette, tips).

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### 3. Methods

#### 3.1. General Procedures for Mother Plants

An important step in the establishment of in vitro regeneration protocols is the choice and maintenance of the mother plant in adequate nutritional and sanitary conditions. For *A. sellowiana*, selected mother plants are cultivated in pots with 50 L capacity, containing PlantMax<sup>®</sup> as substrate. The plants are maintained in a Phytotron at 25 ± 2 °C under 16 h photoperiod, and 200 μmol/m<sup>2</sup>/s photosynthetic photon flux supplied by cool-white fluorescent lamps (Sylvania<sup>®</sup>) and high pressure sodium vapor lamps (Empalux<sup>®</sup>—VST) (see Fig. 2a, b). The plants are watered every day and irrigated monthly with a commercial nutritional solution.

#### 3.2. Organogenesis

##### 3.2.1. Culture Conditions

1. WPM or MS (17) basal media are supplemented with Morel's vitamins or Staba vitamins and 3% sucrose.
2. Add other components, such as growth regulators, as required. Add the correct volume of growth regulators from 1,000 μM stock solutions to obtain the required final concentrations (see Note 1).
3. Adjust pH to 5.8 using 0.5 N HCl or 0.5 N NaOH.
4. Add 0.65% Difco Agar.
5. Sterilize the media by autoclaving at 121 °C for 15 min.
6. Store the autoclaved media at room temperature in the dark for a maximum of 1 month.
7. The cultures are maintained in culture room at 25 ± 2 °C, photosynthetic photon flux of 40 μmol/m<sup>2</sup>/s, 16 h photoperiod, and relative humidity of 60 ± 5%.

##### 3.2.2. Shoot Regeneration

1. Two months before initiation of in vitro cultures, selected mother plants (see item 3.1) are drastically pruned to promote the new growth. Additionally, the pruned plants are sprayed with 0.1% Benlate<sup>®</sup>.

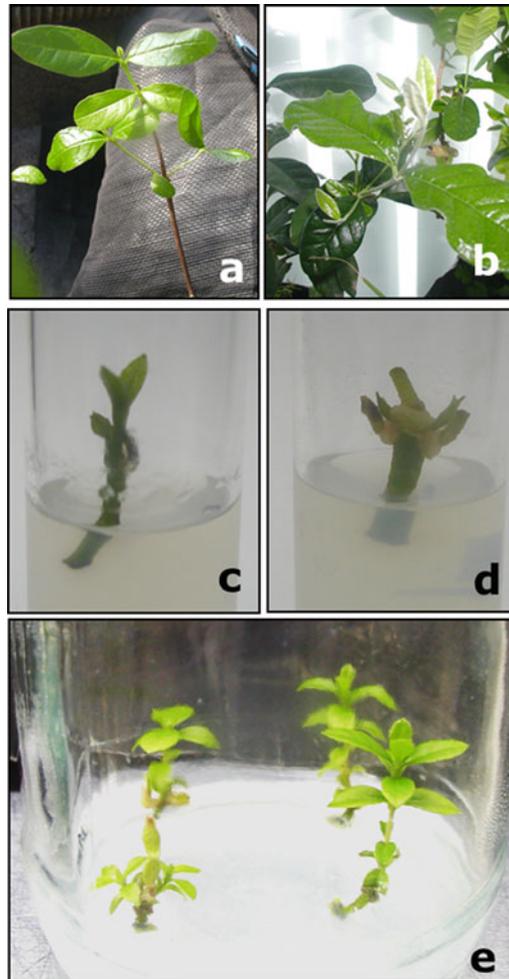


Fig. 2. Nodal cultures of *A. sellowiana*: (a) mother plants kept in chamber room with 16 hour photoperiod photosynthetic photon flux of  $100 \mu\text{mol}/\text{m}^2/\text{s}$  and  $60 \pm 5\%$  relative humidity. Note the new elongated sprouts with light green leaves; (b) mother plants kept in chamber room with 16 hour photoperiod photosynthetic photon flux of  $200 \mu\text{mol}/\text{m}^2/\text{s}$  and  $60 \pm 5\%$  relative humidity. Note the full expanded dark green leaves; (c, d) microcutting in WPM medium after 15 days; and (e) after 30 days.

2. One month before the in vitro introduction, selected mother plants are transferred to Phytotron<sup>®</sup> at  $25 \pm 2 \text{ }^\circ\text{C}$  under 16 h photoperiod, and  $100 \mu\text{mol}/\text{m}^2/\text{s}$  photosynthetic photon flux supplied by cool-white fluorescent lamps (Sylvania<sup>®</sup>) and high pressure sodium vapor lamps (Empalux<sup>®</sup>—VST). Reduce light intensity to stimulate etiolation of new sprouts. The matrix plants were sprayed three times every 5 days with 1 g/L Benlate<sup>®</sup>.
3. Collect sprouts with two pairs of leaves and the apical meristem, place in sealed plastic bags, and transport immediately to the laboratory.

4. Wash them in tap water for 10 min.
5. In laminar flow cabinet minicuttings with nodal segments are treated with 70% ethanol for 1 min. In sequence they are submitted to a double disinfection with 5 and 2.5% NaOCl solution and Tween 20, each for 5 min. Rinse explants three times in sterile distilled water containing 250 mg/L citric acid and 250 mg/L ascorbic acid for preventing oxidation.
6. The leaves of the explants are carefully removed with a scalpel.
7. Culture explants in test tubes containing 15 mL basal gelled WPM medium (see Fig. 2c, d).
8. Explants multiply every 30 days by the same medium of induction (see Fig. 2e).

### 3.2.3. Shoot Regeneration from Apical Meristem

1. In laminar flow cabinet, sterile apical buds of 1–1.5 cm long with 70% ethanol for 20 s and after with 1% NaOCl for 10 min. Rinse three times in sterile distilled water.
2. Culture explants in test tubes (22 × 150 mm) containing 15 mL basal gelled MS medium supplement with 0.2% activated charcoal, 2.0 μM BAP, 0.3 μM GA<sub>3</sub>, and 0.05 μM NAA.
3. Subculture every 30 days in the same induction medium.

### 3.2.4. Shoot Regeneration from Nodal Segment

1. Dip minicuttings (3–4 cm) from rejuvenated shoots of selected mother plants in an antioxidant solution (citric/ascorbic acid 360:284 μM).
2. In laminar flow cabinet, sterile minicuttings are dipped in ethanol (70%) for 1 min, and then submitted to a double disinfection with 0.15% HgCl<sub>2</sub>, 1% NaOCl solution, and Tween 20, each for 5 min. Rinse explants three times in sterile distilled water. Reduce microcuttings to 1–1.5 cm long and dip them again in the antioxidant solution.
3. Transfer them to bottles containing 30 mL gelled MS medium supplemented with 13 μM BAP and 0.3 μM GA<sub>3</sub>.
4. Subculture every 30 days to the same culture medium described in Subheading 3.2.4, step 3.

### 3.2.5. Shoot Regeneration from Seedlings

1. In laminar flow cabinet, sterile the seeds with 0.5–1.0% NaOCl for 20 min. Rinse explants three times in sterile distilled water.
2. Germinate sterilized seeds in bottles (300 mL) containing 30 mL basal gelled WPM medium supplemented with 0.15% activated charcoal.
3. The germination is undertaken in culture room at 25 ± 2 °C, with 16 h photoperiod, photosynthetic photon flux of 40 μmol/m<sup>2</sup>/s, and 60 ± 5% relative humidity.
4. Shoots from 6- to 8-week-old seedlings are used to initiate cultures for shoot multiplication.

5. Nodal segments (1–1.5 cm long) are inoculated in test tubes containing 15 mL of basal gelled medium WPM supplemented with 5  $\mu$ M Kin.
6. Alternatively:
  - (a) The culture medium may be PGR free.
  - (b) Two nodal segments (~2.5 cm of long) are inoculated in WPM basal liquid medium with 20  $\mu$ M IBA for 6 days. After induction, the explants are transferred to basal gelled medium WPM without auxin (see Note 2).
  - (c) Nodal segments (1–1.5 cm long) are inoculated in test tubes containing 10 mL of basal medium WPM supplemented with Staba vitamins, 3% sucrose, and gelled with 0.8% Difco Agar (see Note 3).
7. Transfer microcutting containing two nodal segment (~2.5 cm long) to a PGR-free basal solid medium or alternatively, to ex vitro substrate in Phytotron® (see Note 4).

#### 3.2.6. Rooting

1. Subculture shoots with three nodal segments to test tubes containing 15 mL gelled  $\frac{1}{4}$  MS basal medium supplemented with 1.2  $\mu$ M IBA and 0.15% activated charcoal, and grow up to 30 days.
2. After 30 days, transfer plantlets to test tubes containing 15 mL basal gelled MS medium with 0.15% activated charcoal.

#### 3.2.7. Plantlet Acclimatization

1. Plantlets are transferred to trays with 72 cells filled with PlantMax® after 30 days of rooting. The trays are then placed inside a plastic box covered with glass to keep a saturated humidity microenvironment.
2. The trays are placed in Phytotron® adjusted to  $27 \pm 2$  °C, photosynthetic photon flux of 200  $\mu$ mol/m<sup>2</sup>/s, 16 h photoperiod, and relative humidity of  $80 \pm 5\%$ .

### 3.3. Somatic Embryogenesis

#### 3.3.1. Culture Conditions

1. The basal LPM is supplemented with Morel's vitamins. Add 0.2% (w/v) Phytigel®.
2. Follow all other steps according to Subheading 3.2.1.
3. Maintain cultures in the dark culture room at  $25 \pm 2$  °C during the induction phase.

#### 3.3.2. Somatic Embryogenesis from Immature Zygotic Embryos

1. Wash immature fruits with water and commercial neutral soap and remove fruit epicarp.
2. In laminar flow chamber, the seeds are disinfested with 70% ethanol for 1 min; 1% NaOCl for 20 min, and then rinse three times in sterile distilled water.
3. With the aid of a stereomicroscope, excise immature zygotic embryos and inoculate them in test tubes containing 20 mL

basal solidified LPm supplemented with 10  $\mu\text{M}$  2,4-D and 4 mM Gln. The cultures are maintained in the culture room at 23 °C in the dark.

4. After 120 days in culture, isolate and transfer somatic embryos (SE) ranging from the torpedo to the cotyledon stages, to half-strength MS medium, containing 0.5  $\mu\text{M}$  BAP. During the maturation and conversion stages of SE, keep the cultures in culture room with a photosynthetic photon flux 40  $\mu\text{mol}/\text{m}^2/\text{s}$  provided by cool white lamps and a 16 h photoperiod at 23 °C.

### 3.3.3. Somatic Embryogenesis from Mature Zygotic Embryos

1. Place mature seeds in 0.5% NaClO solution for overnight.
2. In laminar flow cabinet, the seeds are disinfested with 1% NaOCl for 20 min, and then rinse three times in sterile distilled water (see Fig. 3a).
3. Remove two external integuments of seed with scalpel under the stereomicroscope (see Fig. 3b).
4. Culture zygotic embryos in test tubes containing 15 mL basal gelled LPm supplemented with sucrose 3% 20  $\mu\text{M}$  2,4-D, 0.1 g/L myo-inositol, and 8 mM Glu.
5. Uncoil of zygotic embryos is observed after 5 days (see Fig. 3c; Note 5).
6. The identification of embryogenic cultures is performed by the double staining with acetic carmine and Evan's Blue technique, according to Gupta and Durzan (25) (see item 3.3.5; Fig. 3d).
7. After 30 days, globular stage somatic embryos are observed on the surface of the cotyledon leaf (see Fig. 3e).
8. The subsequent developmental stages, heart, torpedo, and cotyledon, are observed after 45 days in culture. An induction rate of 100% can be obtained after 90 days in culture (see Fig. 3f).

### 3.3.4. Somatic Embryogenesis from Stamens

1. Floral buds of the *A. sellowiana* are collected before anthesis and sterilized as described in item 3.2.3.
2. After dissecting the bud tissues in aseptic chamber, the stamens are excised and inoculated in test tubes containing 15 mL of basal gelled medium LPm supplemented with sucrose 3% 4 mM Gln, 10  $\mu\text{M}$  Picloram, and 1  $\mu\text{M}$  Kin. Cultures are kept in culture room, in the dark at 27 °C for 60 days.
3. Embryogenic calluses are transferred to Petri dishes (15  $\times$  100 mm) containing 25 mL of basal gelled culture medium LPm sucrose 3% supplemented with 5  $\mu\text{M}$  Picloram and 1  $\mu\text{M}$  Kin. Cultures are kept in culture room in the dark at 27 °C for 60 days.
4. Embryogenic calluses are subcultured to Petri dishes containing 25 mL of basal gelled LPm culture medium supplemented

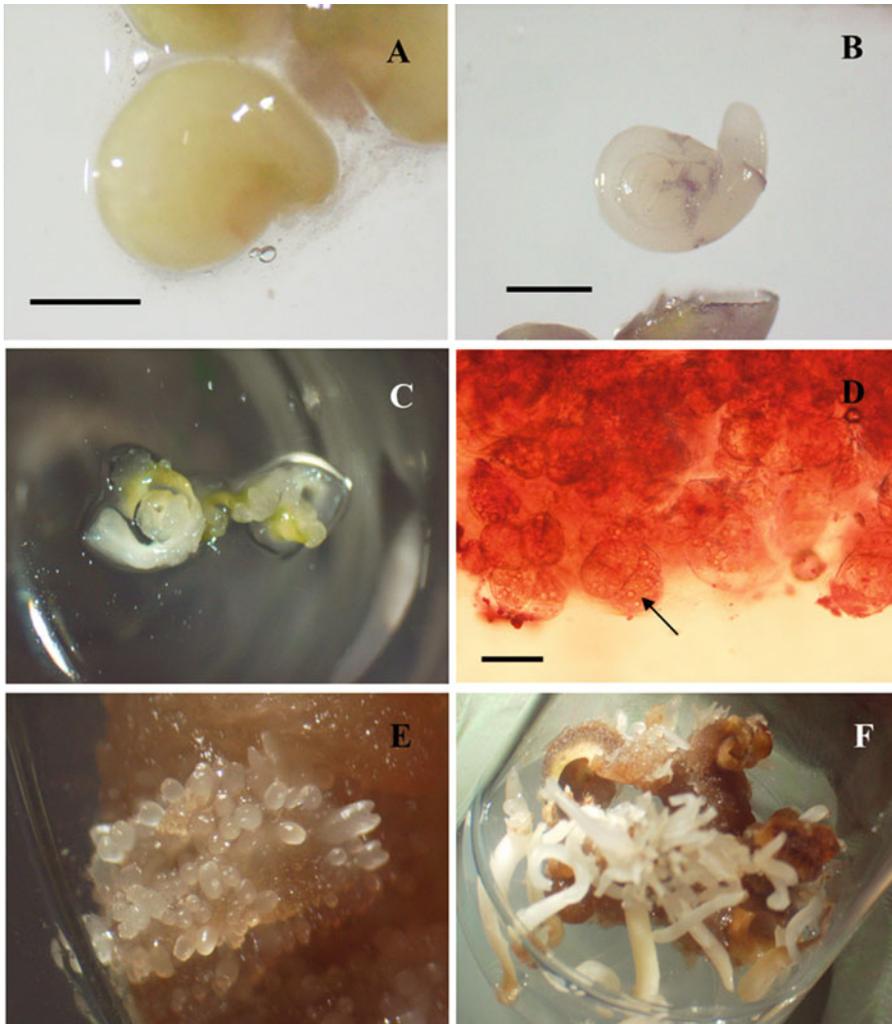


Fig. 3. Induction and development of *A. sellowiana* somatic embryogenesis: (a) seeds extracted from mature fruits; (b) excised embryos after the integuments are removed; (c) uncoil of zygotic embryos after 5 days in culture; (d) embryogenic cells stained with acetocarmine after 15 days in culture (arrow: somatic pro-embryo); (e) globular and heart-staged somatic embryos on the surface of cotyledonal leaf after 30 days; and (f) Torpedo and cotyledonary-staged somatic embryos after 90 days in culture. Bars: a, b: 0.1 cm; d: 100  $\mu\text{m}$ .

with 1  $\mu\text{M}$  Picloram and 0.5  $\mu\text{M}$  Kin. The cultures are kept in culture room at 27  $^{\circ}\text{C}$  during 60 days.

5. For the scaling-up of suspension cultures, transfer 1 g fresh mass of embryogenic culture in 1,000 mL nipple-flasks, containing 100 mL basal liquid LPm supplemented with sucrose 3% 1  $\mu\text{M}$  2,4-D and 1  $\mu\text{M}$  2-iP.
6. The nipple-flasks are placed on a rotating orbital shaker (Steward Apparatus) at 1 rpm and incubated at 27  $^{\circ}\text{C}$  in the dark.
7. Cell fractions higher than 74  $\mu\text{m}$  are collected using stainless steel tissue sieves (Celetor apparatus, Sigma) and re-inoculated

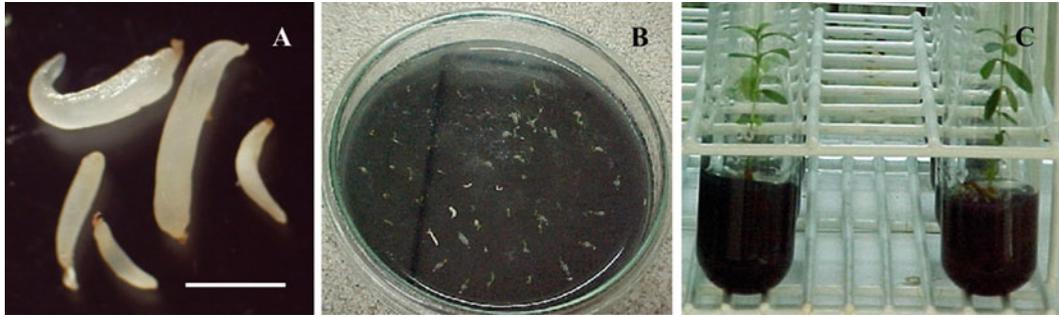


Fig. 4. Conversion of *Acca sellowiana* somatic embryos. (a) Somatic embryos torpedo-staged (Bar, 0.25 cm); (b) the somatic embryos, inoculated in Petri dishes containing basal gelled medium LPm supplemented with 0.15% activated charcoal, 0.5  $\mu\text{M}$  BAP, and 1  $\mu\text{M}$  GA<sub>3</sub>; (c) seedlings after 60 days.

on Petri dishes, containing 25 mL of gelled LPm PGR-free culture medium.

8. For the maturation step, the cultures are subcultured to Petri dishes containing 25 mL of gelled LPm PGR-free culture medium sucrose 3%.

### 3.3.5. Cytochemical Procedures

1. The quality of cultures is evaluated by double staining under light microscope based on acetocarmine and Evan's Blue staining (25).
2. An aliquot of cells is placed on a watch glass.
3. A drop of 1% acetocarmine (w/v) (see Note 6) is added to the sample for 1 min.
4. Excess is removed with the aid of toilet paper.
5. Drop 0.05% Evans Blue (w/v) (see Note 7) for 1 min.
6. Remove again the excess with the aid of toilet paper.
7. Drop 1 mL of sterile distilled water.
8. Drop with a pipette an aliquot on a slide glass, then visualize in the light microscope (see Note 8; Fig. 3d).

### 3.3.6. Conversion of Somatic Embryos to Plantlets

1. Select torpedo-staged somatic embryos (see item 3.3.3) for conversion to seedlings (see Fig. 4a).
2. Inoculate them in Petri dishes containing 25 mL of basal gelled medium LPm supplemented with 3% sucrose 0.15% activated charcoal, 0.5  $\mu\text{M}$  BAP, and 1  $\mu\text{M}$  GA<sub>3</sub> (see Fig. 4b) for 30 days.
3. The converted plantlets are inoculated in test tube containing 15 mL of basal gelled LPm culture medium supplemented with 3% sucrose and 0.15% activated charcoal (see Fig. 4c).
4. The plantlets are maintained in culture room with the photo-synthetic photon flux of 40  $\mu\text{mol}/\text{m}^2/\text{s}$  provided by cool white lamps, relative humidity of  $60 \pm 5\%$ , and 16 h photoperiod.

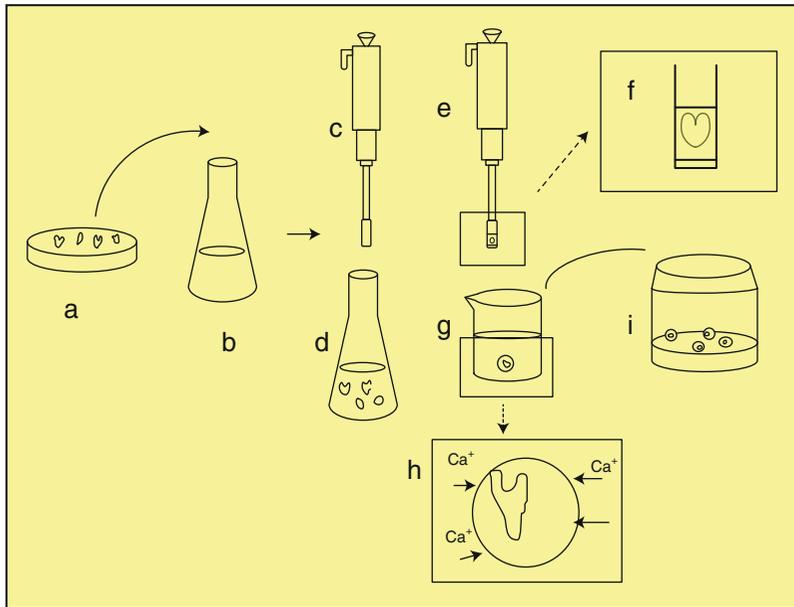


Fig. 5. Schematic illustration for *A. sellowiana* synthetic seed production. (a) Petri dish containing torpedo and cotyledonary-staged somatic embryos; (b) 1% sodium alginate (w/v) diluted in LPm culture medium (gelled matrix); (c) pipette; (d) somatic embryos dipped in 1% sodium alginate (w/v) diluted in LPm culture medium (gelled matrix); (f) capture of somatic embryos embedded in the gelled matrix; (g) which are dipped in a 50 mM  $\text{CaCl}_2$  solution; (h) detail of capsule complexation; and (i) conversion of synthetic seeds.

### 3.4. Synthetic or Artificial Seeds

Synthetic, artificial, or somatic seeds are analogous seeds to the true or botanical seed. Each somatic embryo is encapsulated in a calcium alginate bead, which protects against mechanical damages during the storage and sowing (26). In order to improve the conversion of the synthetic seeds, the supplementation of nutrients has been evaluated, including PGRs and addition of other substances to the alginate matrix (18, 20).

Basically, a mixture of somatic embryos and sodium alginate is used for the encapsulation. Pick up a single somatic embryo together with sodium alginate with an Eppendorf pipette tip and release in  $\text{CaCl}_2$  solution. For germination, immerse synthetic seeds in  $\text{KNO}_3$  (100–200 mM) solution for 2 min to soften the capsule (see Fig. 5).

#### 3.4.1. Synthetic Seeds from Somatic Embryogenesis

1. Select torpedo-staged somatic embryos (see item 3.3.3; Fig. 4a).
2. Preconvert them in Petri dishes containing 25 mL of basal gelled medium LPm supplemented with 0.15% activated charcoal, 0.5  $\mu\text{M}$  BAP, 1  $\mu\text{M}$   $\text{GA}_3$ , and sucrose 3% (Preconversion medium), for 15 days before encapsulation (see Fig. 4b).
3. The synthetic seeds are established by capturing preconverted somatic embryos in a capsule with 1% sodium alginate diluted in the medium  $\frac{1}{2}$  LPm salts supplemented with 0.15% activated charcoal, 0.5  $\mu\text{M}$  BAP, 1  $\mu\text{M}$   $\text{GA}_3$  (see Fig. 6a), and then

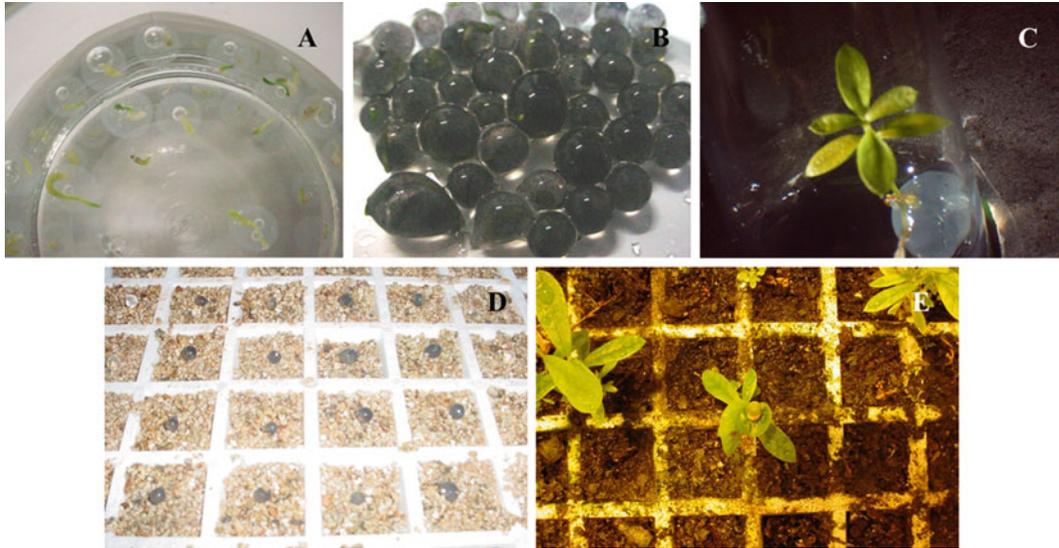


Fig. 6. *Acca sellowiana* synthetic seeds. (a) Preconverted somatic embryos complexed with 1% sodium alginate in solution of 50 mM  $\text{CaCl}_2$  for 20 min; (b) synthetic seeds with 1.5% activated charcoal; (c) conversion of synthetic seeds after 30 days; (d) ex vitro conversion of synthetic seeds placed into trays containing as substrate a mixture of vermiculite and Plantmax® 1:1 (v:v), and (e) acclimatization of plantlets derived from synthetic seeds.

releasing the capsule in a solution of 50 mM  $\text{CaCl}_2$  for 20 min (see Fig. 6b).

4. The capsules containing preconverted embryos are placed in bottles (200 mL) containing 30 mL of water gelled with 0.7% Difco Agar (see Fig. 6c).
5. The synthetic seeds are maintained in culture room at  $25 \pm 2$  °C with the photosynthetic photon flux of  $40 \mu\text{mol}/\text{m}^2/\text{s}$  provided by cool white lamps, relative humidity of  $60 \pm 5\%$ , and 16 h photoperiod.
6. Alternatively the capsules are sowed into trays with 72 cells ( $16 \text{ cm}^3$  each) containing a mixture of vermiculite and Plantmax® 1:1 (v:v) and then maintained in acclimatization room (see Fig. 6d, e).
7. The trays are kept in Phytotron® adjusted to  $25 \pm 2$  °C, 16 h photoperiod with a photosynthetic photon flux of  $200 \mu\text{mol}/\text{m}^2/\text{s}$ . The trays are placed inside a plastic box covered with glass to allow light entry and to reduce water exchange. Wet periodically the plantlets germinating capsules with a 20% solution of LPM salts (see Note 7).

### **3.5. Liquid Culture in Temporary Immersion System**

A temporary immersion system (RITA®; recipient for automated temporary system) is a bioreactor, developed by Teisson and Alvard (27). This system is relatively simple and easy to use, allowing contact between all parts of the explants with the nutrient solution,



Fig. 7. A temporary immersion system RITA® developed by Teisson and Alvard (27).

together with the renewal of the atmosphere by ventilation of culture promoted by the air compressor (see Fig. 7). Several authors reported many advantages including improved micropropagule quality, reduced consumables costs, reduced labor costs (28, 29), better leaf development, and reduced hyperhydricity (30). Further, plants from the temporary immersion system have been found to be more suitable for acclimatization and development towards photoautotrophy (29).

#### 3.5.1. Conversion of Somatic Embryos in System RITA®

1. Embryogenic cultures (see item 3.3.3), containing embryos in different developmental stages are inoculated in RITA® apparatus containing 200 mL of basal liquid medium LPm supplemented with sucrose 3% 0.5  $\mu\text{M}$  BAP, 1  $\mu\text{M}$  GA<sub>3</sub>, and 0.05  $\mu\text{M}$  Fluridone.
2. The cultures are kept in this condition during 30 days for the conversion of embryos into plantlets.
3. After the plantlets are inoculated in test tube containing 15 mL of LPm basal medium, supplement with 3% sucrose, 0.15% activated charcoal, and gelled with 0.7% Difco Agar.
4. The plantlets are maintained in culture room at  $25 \pm 2$  °C with 40  $\mu\text{mol}/\text{m}^2/\text{s}$  photosynthetic photon flux of provided by cool white lamps, relative humidity of  $60 \pm 5\%$ , and 16 h photoperiod.

## 4. Notes

1. Remember  $0.001\text{ M} = 1\text{ mM} = 1,000\ \mu\text{M}$ . To determine how many g/L are needed for  $0.001\text{ M}$  concentration, first, look up the molecular weight of the PGR. Consider as example the Kinetin. Its molecular weight is 215.2, then a  $0.001\text{ M}$  solution ( $1,000\ \mu\text{M}$ ) consists of  $0.2152\text{ g/L}$  of solution.

$$1,000\ \mu\text{M} = 0.001\text{ M}$$

$$M = \frac{m_1}{V_1 \times \text{mol}}$$

$M$  = molarity (in g/L);  $m_1$  = mass of solute (in grams); mol = molar mass of solute;  $V_1$  = liter of solution.

$$0.001\text{ g/L} = \frac{m_1}{1\text{L} \times 215.2} \therefore m_1 = 0.2152\text{ g}$$

2. After 30 days the mean microcutting rooting is 68.9%.
3. After 15 days, the microcuttings are subcultured to the same medium composition.
4. Select microcuttings with two nodal segments ( $\sim 2.5\text{ cm}$  long). The basal portion of microcuttings is treated with  $100\ \mu\text{M}$  IBA for 60 min and transplanted into trays containing substrate composed of a mixture of vermiculite and carbonized rice coat 1:1 (v:v). During acclimatization phase, the plantlets should be sprayed every week with a solution of  $\frac{1}{4}$  WPM salts. Thirty days in this condition the plantlets are  $45.3\text{ mm}$  long with a mean root number of 11.3.
5. The zygotic embryos are excised in aseptic chamber and inoculated in test tubes containing  $15\text{ mL}$  of basal gelled medium LPm supplemented with  $20\ \mu\text{M}$  2,4-D and  $4\text{ mM}$  Gln. After 2 weeks the cultures are transferred to the basal medium LPm PGR-free. The induction of somatic embryogenesis occurs directly from the cotyledonary surface of the zygotic embryos starting from 30 to 45 days. Two weeks of 2,4-D shock results in 93.7% of embryogenic induction after 12 weeks.
6. Acetocarmine 2%: Dissolve  $2\text{ g}$  carmine in  $100\text{ mL}$  acetic acid 45% (v/v). Boil in reflux condenser by 3 h. Cool in room temperature and filter.
7. To Evan's Blue: Dissolve  $1\text{ g}$  Evan's Blue in  $100\text{ mL}$  distilled water.
8. *Comment:* small isodiametric cells with high nucleoplasmic, few small vacuoles stained in red are embryogenic cells.

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## Micropropagation of *Vaccinium* sp. by In Vitro Axillary Shoot Proliferation

Wojciech Litwińczuk

### Abstract

The *Vaccinium* genus contains several valuable fruit and ornamental species, among others: highbush blueberry (*Vaccinium* × *corymbosum* L.), cranberry (*Vaccinium macrocarpon* Ait.), and lingonberry (*Vaccinium vitis-idaea* L.). In some most popular and valuable cultivars, the conventional propagation methods, exploiting hard or soft wood cuttings, are inefficient. The demand for nursery plants could be fulfilled only by micropropagation. In principle cultivars are propagated in vitro through similar three-stage method, based on subculture of shoot explants on different culture media supplemented with IAA (0–4 mg/L) and 2iP (5–10 mg/L), and rooting shoots in vivo. The obtained plantlets are transferred to peat substrate and grown in the glasshouse until the end of growing period. The development of adventitious shoots should be monitored and controlled during in vitro stages. Many clones have specific requirements for growing conditions and/or are recalcitrant.

**Key words:** Adventitious shoots, Axillary shoots, Blueberry, Cranberry, Lingonberry, Nodal explants

---

### 1. Introduction

Highbush blueberry (*Vaccinium* × *corymbosum* L., syn. *Vaccinium corymbosum* hort. non L., *Vaccinium* × *covilleianum* But. et Pl.) is relatively a new fruit crop when compared to other fruit species. The first valuable cultivars (“seven big”) were introduced in 1950s. It is a hybrid of at least three species (*Vaccinium australe* Small, *V. corymbosum* L., and *V. angustifolium* Ait.) (1). The *Vaccinium* genus also contains other useful species among others: cranberry (*Vaccinium macrocarpon* Ait.), lingonberry (*Vaccinium vitis-idaea* L.), and bilberry (*Vaccinium myrtillus* L.). The commercial world production of blueberries is 214–331 thousand tons, grown in the area 48.3–74.0 thousand hectares during the period 1999–2008 (FAOSTAT). The analogous production of cranberries was 286–440

thousand tons on 20.2–22.6 thousand hectare area (ibid). Several species of *Vaccinium* are highly valuable for their edible fruits, which are excellent source of health-promoting nutrients, among others: dietary fibers, antioxidants (vitamin C, beta-carotene, folic acid, ellagic acid, anthocyanins), and antibacterial and antifungal agents, like anthocyanosides, benzoic acid. The berries are consumed either as fresh or processed products such as frozen, canned, liquid, concentrates, and dried goods, like preserves, candies, jells, juices, syrups, ice creams, pastries, muesli, milk and alcohol beverages, as well as nutritional supplements and pharmaceuticals. There is an increase in highbush berries demand and fetch attractive price, and that has rapidly expanded its cultivation area worldwide in the last decades of twentieth century. In order to meet the increasing demand of blueberry plantlets, micropropagation would be the most appropriate approach. The micropropagation is the only method for rapid propagation of elite breeding clones and new cultivars. By the end of 1980s more than one million highbush blueberry plants were propagated annually worldwide (2). It should be noted that at the same time some controversies about value of micropropagated plants, among others of *Ericaceae* family, appeared (3, 4) and, although rare, remain until now. They were based on some sporadic but confusing mentions of excessive vegetative growth (too many thin lateral shoots), delayed fruit harvest for some years, or smaller berries. However, despite of it at the turn of the twentieth/twenty-first century, micropropagated plants prevailed conventional ones in nurseries. The first articles about micropropagation of *Vaccinium* sp. appeared at the turn of the 1970/1980s. Since then numerous studies regarding blueberry (*Vaccinium* sp.) in vitro cultures have been published. In fact, both blueberries and cranberries are micropropagated with similar procedure—subculture of shoot explants on the medium supplemented with indole-3-acetic acid (IAA, 0–4 mg/L) and 6- $\gamma$ , $\gamma$ -dimethylallylaminopurine (2iP, 5–10 mg/L). However, intra- and inter-species differ greatly in the response of *Vaccinium* sp. cultivars to the type and concentration of cytokinins, auxins, and Fe salts (see Fig. 1). Therefore, it is recommended to optimize culture conditions for each genotype separately. Also, explants easily form adventitious shoots, even during initiation stage of *Vaccinium* in vitro cultures (5–9). As adventitious shoots are better habituated to in vitro conditions than axillary shoots, they could rapidly (in the few passages) displace axillary shoots from micropropagation procedure. Thus, the special care should be taken to prevent such phenomena as adventitious shoots of many species are suspected to be the main source of somaclonal variation. The in vitro culture techniques are also utilized in cryopreservation (10), in vitro selection (11), mutagenesis (12), interspecific and intersectional hybrids (13), genetic transformation (3, 14, 15), and production of secondary metabolites of pharmaceutical value in suspension and callus cultures (16, 17).

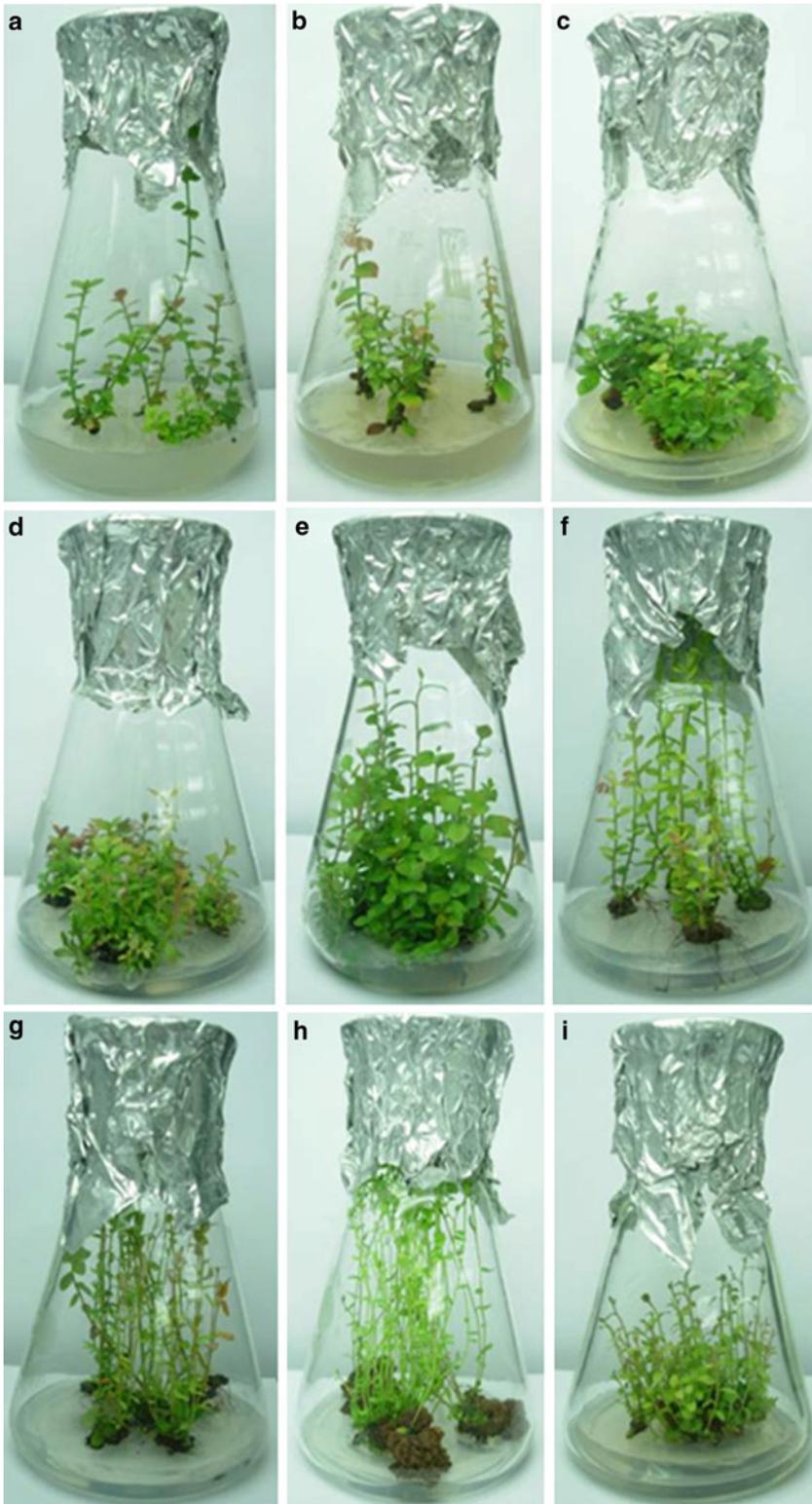


Fig. 1. Comparison of the growth of *Vaccinium* sp. in vitro cultures on the same medium. (a) highbush blueberry 'Bluecrop', (b) highbush blueberry 'EarlyBlue', (c) highbush blueberry 'Blueray', (d) highbush blueberry—false 'Bluecrop', (e) highbush blueberry 'Herbert', (f) cranberry 'Stevens', (g) cranberry 'Pilgrim', (h) *Vaccinium microcarpon* 'nn', (i) lingonberry 'Runo Bielawskie'.

In this chapter, the protocol is described for axillary shoot initiation and multiplication *in vitro*, with subsequent rooting shoots *in vivo* along with acclimatization of regenerated plants.

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## 2. Materials

### **2.1. Surface Sterilization of Source Material**

1. Ethanol 70% (v:v).
2. Commercial bleach solution (e.g. “ACE” bleach; 5% (v:v) NaClO), diluted 1:5 (v:v) with tap water or solution of 0.1% mercuric chloride.
3. Distilled (or autoclaved reverse-osmosis) water (150 mL aliquots in 300 mL screw capped jars).
4. Laboratory shaker or ultrasonic washer.
5. Potted parental plant as a source of explants.
6. Solutions of Bayleton 5 WP (1.0 g/L), Topsin M (1.5 g/L), or equivalent fungicides to spray parental plants.

### **2.2. Tissue Culture Facilities and Culture Media**

1. Labwares: autoclave, magnetic stirrer with heating, dry-heat sterilizer, microwave oven, pH meter, refrigerator, horizontal laminar flow bench.
2. Instruments: scalpels, forceps, glass bead sterilizers, or gas burners.
3. Sterile Petri dishes (100 × 15 mm) or sterile paper sheets (keep at 160°C for minimum 2 h) to prepare explants.
4. Sterile dispensers of liquid medium to make double-phase medium, like graduated (25 mL) pipettes with pumps, autoclavable and adjustable (1–5 mL) single-channel pipettes.
5. The media for (a, b) culture initiation, and (c, d, e) shoot proliferation (see Tables 1 and 2).
6. 1 M HCl and 1 M NaOH.
7. Test tubes, 15–75 mL capacity, with caps (16 × 75, 25 × 150 mm) or 25–50 mL conical Erlenmeyer’s flasks.
8. Aluminum packaging foil (0.04 mm thick) for culture initiation and first subcultures.
9. 300–500 mL glass jars closed with autoclavable, transparent lids (or adequate culture vessels) for shoot proliferation and rooting.
10. Plastic, thermostable (0.1–3 L) beakers.
11. Culture room with air conditioning. The possibility of temperature (22–28°C), light intensity (30–50  $\mu\text{mol}/\text{m}^2/\text{s}$  PPF), and photoperiod (16/8 h day/night) regulation is advantageous. The white growth shelves with white fluorescent lamps, preferentially with bottom-cooling system.

**Table 1**  
**Composition of media used for micropropagation of *Vaccinium* sp.**

Stages	(a) Initiation (liquid)	(b) Initiation (solidified)	(c) Proliferation (solidified)	(d) Proliferation—last passage (solidified)	(e) Proliferation—last passage (liquid)
Macronutrients	10–50% BM <sup>a</sup>	10–50% BM	100% BM	100% BM	100% BM
Micronutrients <sup>b</sup> (without Fe salts)	50% MS	50% MS	100% MS	100% MS	100% MS
FeNaEDTA+FeEDDHA	36.7 + 5 mg/L	36.7 + 5 mg/L	58.7 + 10 mg/L	58.7 + 10 mg/L	58.7 + 10 mg/L
Vitamins <sup>c</sup>	100% WPM	100% WPM	100% WPM	100% WPM	100% WPM
<i>Myo</i> -inositol	100 mg/L	100 mg/L	100 mg/L	100 mg/L	100 mg/L
Sucrose	15 g/L	15 g/L	30 g/L	30–40 g/L	30–40 g/L
Cytokinins <sup>d</sup> (ZEA/2iP/AS)	0.5–1/80 mg/L	0.5–1/80 mg/L	–/5–10/80 mg/L	–/5–10/80 mg/L	–
Auxins <sup>d</sup>	IAA 1.0 mg/L or IBA 0.2 mg/L	IAA 1.0 mg/L or IBA 0.2 mg/L	IAA or IBA 0–1.0 mg/L	–	–
Agar (see Note 9)	–	5–6 g/L	6–11 g/L	6–11 g/L	–
Other ingredients	fructose (5 g/L), (PVP 360, 100 mg/L), L-cysteine 5 mg/L, PPM™ (0.5 mL/L),	fructose(5 g/L), (PVP 360, 100 mg/L), L-cysteine 5 mg/L, PPM™ (0.5 mL/L),	Fe-EDDHA 10 mg/L	Fe-EDDHA 10 mg/L	Fe-EDDHA 10 mg/L
pH	5.0	5.0	5.0	5.0	5.0

<sup>a</sup>BM—one of media recommended for micropropagation of *Vaccinium* sp (see Note 6)

<sup>b</sup>MS micronutrients: NaFeEDTA 36.7 mg/L, KI 0.83 mg/L, H<sub>3</sub>BO<sub>3</sub> 6.3 mg/L, MnSO<sub>4</sub> × 4H<sub>2</sub>O 22.3 mg/L, ZnSO<sub>4</sub> × 7H<sub>2</sub>O 8.6 mg/L, Na<sub>2</sub>MoO<sub>4</sub> × 2H<sub>2</sub>O 0.25 mg/L, CuSO<sub>4</sub> × 5H<sub>2</sub>O 0.025 mg/L, CoCl<sub>2</sub> × 6H<sub>2</sub>O 0.025 mg/L

<sup>c</sup>Woody Plant Medium (WPM, (23)) vitamins: glycine 2.0 mg/L, thiamine HCl 1.0 mg/L, pyridoxine HCl 0.5 mg/L, nicotinic acid 0.5 mg/L

<sup>d</sup>Cytokinins, auxins (see Notes 8 and 11)

**Table 2**  
**Composition of macronutrients in media used in in vitro cultures of *Vaccinium* sp.**

Compounds (mg/L)	AND_M (20)	AND_Z (21)	EC (22)	PMN (19)	WPM (23)	Z-2 (5)	DR (24)
NH <sub>4</sub> NO <sub>3</sub>	400	400	400	480	400	160	550
KNO <sub>3</sub>	190	480	202	305	–	202	330
K <sub>2</sub> SO <sub>4</sub>	–	–	–	–	990	–	140
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	–	–	132	–	–	198	50
Ca(NO <sub>3</sub> ) <sub>2</sub> × 4 H <sub>2</sub> O	684	–	–	470	556	708	410
CaCl <sub>2</sub> × 2 H <sub>2</sub> O	–	440	440	–	–	–	200
KH <sub>2</sub> PO <sub>4</sub>	370	–	408	–	170	408	220
KH <sub>2</sub> PO <sub>4</sub> × H <sub>2</sub> O	–	–	–	205	–	–	–
NaH <sub>2</sub> PO <sub>4</sub> × H <sub>2</sub> O	–	380	–	180	–	–	100
MgSO <sub>4</sub> × 7 H <sub>2</sub> O	370	370	370	370	370	370	370
Na <sub>2</sub> EDTA	74.6	37.2	–	73.2	74.6	74.4	–
FeSO <sub>4</sub> × 7 H <sub>2</sub> O	55.6	27.9	–	60.0	55.6	55.7	–
NaFeEDTA	–	–	56	–	–	–	37.3

### 2.3. Acclimation of Regenerated Plants to Ex Vitro Conditions

1. Greenhouse or plastic tunnel facilities or as a last resort well separated culture room with air conditioning, provided with high pressure sodium lights (60–200 μmol/m<sup>2</sup>/s PPF).
2. Mist chambers (95% RH), multicell plug trays with transparent plastic covers/lids. Cell dimensions about 2 × 3 cm (width, depth), transparent plastic foil.
3. Peat and sand mixture (2:1; v:v) adjusted to pH 4.0 with calcium carbonate, watered with solution of SCOTT'S Peters Plant Starter (10+52+10; 0.8 g/L) and fungicide Previcur 607 SL (1.0 g/L) or equivalent chemicals.
4. Solution of Rovral Flo 255 SC (1.0 g/L) or equivalent fungicide to spray microplants.

## 3. Methods

Micropropagation of *Vaccinium* sp. is carried out with a three-stage method. Different media, elaborated for ericaceous plants, are used to establish, maintain, and multiply cultures (see Tables 1 and 2). Add zeatin (0.5–1 mg/L) for culture initiation in the first

2–3 passages. Replace it with 2iP (5–10 mg/L) in the subsequent 6–8-weeks long passages. In the case of recalcitrant clones do it gradually. The addition of IAA (1–4 mg/L) in the culture medium is not necessary. The double-phase medium is recommended in the last passage before shoots differentiate roots. The shoots are generally rooted *in vivo* in substrates based on peat, and the obtained plantlets are grown in the greenhouse until the end of growing period. However, it should be mentioned that many clones have specific requirements for culture conditions, and still even some remain recalcitrant. A great attention is needed to prevent overgrowth of the developing adventitious shoots in order to reduce the risk of somaclonal variation.

### **3.1. Preparation and Sterilization of Culture Media**

1. Prepare medium from stock solutions and reverse-osmosis (or distilled) water. Add medium-specific substances and fix the final volume with water. Adjust pH 5.0 with the addition of 1 M HCl and 1 M NaOH before addition of agar.
2. Dispense melted agar into suitable containers, e.g. 2.5–10 mL aliquots into 15–75 mL test tubes for culture initiation, 50 mL aliquots into 300 mL jars for shoot multiplication and rooting, or 100 mL (without agar) into 250–300 mL screw capped jars to prepare double-phase medium.
3. Sterilize vessels with media by autoclaving at 121°C for 15, 20, or 25 min.
4. Store the media in the refrigerator up to 2 weeks.

### **3.2. Plant Source Material and Surface Sterilization**

1. Take 1–2-year-old cranberry and lingonberry plants, 2–3-year-old blueberry plants from nursery and replant them into plastic pots (1–3 L volume) filled with peat mixture designed for growing ericaceous plants. The young rooted cuttings with healthy sprouts may also be used (see Notes 1–3). Place plants in greenhouse or culture room at room temperature at about 10  $\mu\text{mol}/\text{m}^2/\text{s}$  PPFD to obtain slightly etiolated shoots. It is also possible to collect shoots from parental plants grown in the field (see Notes 3 and 4). Spray shoots with solution of Bayleton 5 WP (1.0 g/L), Topsin M (1.5 g/L), or equivalent fungicide 1 week before disinfection.
2. Tear off 5–10 cm sprouts, remove major part of leaf blade (leave petiole with about 2 mm long remnant of leaf to protect axillary bud).
3. Wash shoots for 10 min under cold, running tap water with detergent; surface sterilize by immersion in 70% (v:v) ethanol for 1 min; keep shaking them in a clean jar containing commercial bleach diluted with water (1:5 v:v) for 20 min.
4. Wash shoots three times with sterile reverse-osmosis water for 5 min.

### **3.3. Culture and Maintenance of Explants**

1. After disinfection, excise nodal sections (3–5 cm long) using a sterile scalpel. Prepare about 50 explants per genotype.
2. Put explants vertically in test tubes filled with liquid initiation (a) medium (see Fig. 2a; Table 1; Notes 5–9).
3. Excise new axillary sprouts which emerged *in vitro*. Prepare explants (nodal sections with or without shoot tip, 6–10 mm long) so as to have about 5 mm of internode under lowest axillary bud. Place them in test tubes or other small vessels filled with semisolid initiation (b) medium (see Table 1). The lowest node of explant should not be immersed in the medium.
4. Transfer explants to the fresh culture medium of the same composition every 4–6 week interval to maintain active growth and select healthy cultures.

### **3.4. Multiplication of Shoots**

1. When well-established cultures are obtained, subculture explants on proliferation (c) medium (see Table 2) every 6–8 weeks. Use 300 mL capacity glass jars closed with transparent lids or adequate culture vessels. Place 12–16 explants in each vessel. Try to put only lower internode in the medium (all axillary buds above it) (see Notes 10 and 11).
2. In the last passage (before rooting) make double-phase medium (d, e media) to stimulate elongation of shoots; pour 5–10 mL liquid (e) medium onto solid one (d) (see Table 1).
3. Grow the cultures at  $26 \pm 1^\circ\text{C}$  and 16 h/8 h day/night photoperiod under cool-white light, 20–70  $\mu\text{mol}/\text{m}^2/\text{s}$  PPFD (depending on micropropagation phase) (see Notes 12 and 13).

### **3.5. Acclimation of Regenerated Shoots to Ex Vitro Conditions**

1. Use healthy-looking shoots (2 cm long) for rooting them *ex vitro* (see Notes 14 and 15).
2. If desirable (see Note 16) dip for a few seconds the bases of microcuttings in water–ethanol (1:1, v:v) rooting indole-3-butyric acid (IBA) (3.0 g/L) solution and put down to peat alone or peat and sand mixture (2:1 v:v; pH 4.0) watered with fertilizer “SCOTT’S Peters Plant Starter” solution (0.8 g/L) and fungicide Previcur 607 SL (0.15%). Spray shoots with solution of fungicide Rovral Flo 255 SC (0.1%). Use multicell plug trays with transparent plastic covers. Grow plants at high air relative humidity (95% RH) in 16 h/8 h day/night photoperiod under sodium light at 60–200  $\mu\text{mol}/\text{m}^2/\text{s}$  PPFD (gradually increased) and at 22–25°C.
3. After about 10 weeks transplant rooted shoots to 1–3 L pots filled with peat enriched with fertilizer designed for blueberries and grow them in unheated greenhouse/tunnel (see Note 17).



Fig. 2. Propagation of *Vaccinium* sp. through in vitro cultures. (a) Initiation of highbush blueberry on the liquid medium, (b) adventitious shoots at the explant base (clump of "madshoots"), (c) adventitious shoots on leaf, (d) adventitious shoots on callus, (e) cultures obtained from explants of adventitious (*left*) and axillary (*right*) origin on proliferation medium, (f) cultures obtained from explants of adventitious (*left*) and axillary (*right*) origin on "blank" medium (without plant growth regulators and vitamins), (g) rooted in vivo microshoots of highbush blueberry, (h) seedlings of highbush blueberry, (i) potted plant of cranberry, (j) potted plant of highbush blueberry, (k) twigs of highbush blueberry treated with dikegulac (1 g/L), (l) untreated twigs of highbush blueberry (control). "Figure 2b, © Copyright by Wydawnictwo Uniwersytetu Przyrodniczego w Lublinie (The University of Life Sciences Publishing Centre) Lublin 2009 (9)".

## 4. Notes

1. Start new cultures from the desirable and healthy cultivars, which are readily available with the breeder or from a reliable nurseryman. It is good to establish own collection of parental plants, isolated from any source of contamination, well protected against pathogens.
2. Establish in vitro cultures of desirable cultivar every 1–2 year. Store the older ones in the darkness at 4°C for minimum 1 year without noticeable loss of viability until the well established, new in vitro cultures are obtained.
3. Take shoots for culture initiation from actively/fast growing parental plants. Therefore, use young plants from the greenhouse or shoots from the field-grown plants taken in late spring.
4. It is possible to collect shoots in winter. However, sometimes it is necessary to keep them at low (–2/+4°C) temperature for several weeks and/or soak in warm water bath (+26°C) for 2 weeks to break dormancy. Before that, clean shoots in tap water and spray with fungicides or even shake them in fungicide solution for several hours. Immerse lower parts of shoots in “forcing medium” containing 8-hydroxyquinoline (140 mg/L), citric acid (210 mg/L), and sucrose (20 g/L). Force shoots to obtain etiolated sprouts in the shade at room temperature.
5. There are big intra- and inter-genus differences in the response of *Vaccinium* (blueberry, cranberry, lingonberry) cultivars on culture conditions, among others: the kind and concentration of cytokinins (2iP, zeatin), auxins (IAA, IBA, none), and Fe salt. The differences in culture growth are presented in Fig. 1. It is suggested to check and choose the optimal medium for each genotype separately.
6. Several media, alone or in mix with Murashige and Skoog (MS, (18)) (1:1; v:v) could be used for micropropagation of *Vaccinium* sp. (5, 19–24) (see Table 2). However, many of them promote relatively more intense development of adventitious than axillary shoots. For some highbush blueberry cultivars the higher ax/adv shoots proportion was obtained on the media described by Eccher et al. (PMN) (19) or Zimmerman and Broome (Z-2) (5).
7. Some cultivars are highly sensitive to the salts present in the culture medium and that kill/damage the tissue and cause browning of surviving tissues especially at the initiation stage. To prevent it, it is better to use 2–10× diluted solution of macronutrients.

8. Several authors (6, 25–28) confirmed that zeatin is more suitable than 2iP for initiation and proliferation of *Vaccinium* sp. cultures. Since zeatin is expensive, use it during initiation and replace with 2iP in successive passages. In the case of recalcitrant clones do it gradually—use zeatin (0.5 mg/L) together with 2iP (4–10 mg/L) for 2–3 passages. The application of zeatin is rather not necessary in the propagation of cranberry.
9. The agar commercial brands differ in gelling strength. The low pH and fructose decrease it. The cultures grow faster but develop much more adventitious and hyperhydrous shoots on semiliquid media.
10. *Vaccinium* explants easily form axillary and at least three kinds of adventitious shoots: leaf, node-adjoin, and base-adjoin shoots (5–9) (see Fig. 2b–e). The first ones are developed directly or indirectly from leaves which touch the medium. Node-adjoin adventitious shoots are developed from area close to node, but not directly from axillary bud. They are not heeled and could be easily detached from initial explant, contrary to axillary shoots. The base-adjoin adventitious shoots originate from small callus formed on wounded tissues and/or leaf remains at the base of initial explant immersed in the medium. The adventitious shoots, especially base-adjoin ones (“madshoots”), are better habituated to in vitro conditions than axillary ones (see Fig. 2e, f). Therefore, micropropagation carried out through routine method, based on subculturing of unselected shoot explants or shoot clumps on MS medium supplemented with IAA (4 mg/L) and 2iP (10–15 mg/L) as well as stimulation of shoot elongation on the blank medium causes, in fact, the propagation of highbush blueberry through highly habituated adventitious shoots (madshoots). Adventitious shoots seem to be the cause of somaclonal variation in many species.
11. Some conditions mentioned below facilitate micropropagation through axillary shoots and counteract the domination of in vitro cultures by highly habituated adventitious shoots. However, none of them guarantees maintaining cultures composed exclusively of axillary shoots without decreasing proliferation ratio.
  - Replacement of IAA (1–4 mg/L) with IBA or melatonin (both at 1.0 mg/L) in the proliferation stages (8, 9).
  - Usage of an IBA (1 mg/L)—application of the medium with doubled Mg<sup>2+</sup> ion content, supplemented with 2iP (5–10 mg/L; lower doses for cranberry) and decreased agar dose (5–6 g/L).
  - IAA usage—use of medium supplemented with 2iP (2–5 mg/L), and riboflavin (B<sub>2</sub>; 1–5 mg/L) or phloroglucinol

(PG; 50–100 mg/L), partial or complete substitution of NaFeEDTA with FeEDDHA (up to 100 mg/L), and increased agar concentration (9–11 g/L).

- Use double-phase medium in the final stage of shoot multiplication (before shoot rooting). Do not use solid medium with significantly lower concentration or devoid of cytokinins, as was recommended previously by many authors.
  - Propagation through 2–4-node explants with long internodes. Avoid node explants with short internodes. Elimination from subculture shoot clumps (use of shoot clumps as explants was recommended previously by several authors) and other off-type shoots such as excessive proliferation, thin shoots, reduced leaves, symptoms of vitrification.
  - Placement of explants turned upside down in the medium.
  - Shortening the subculture to the stage of slowing down the growth of axillary shoots and formation of clumps of adventitious shoots at the explant base.
12. Fluorescent tubes, like “aquarelle,” “fluora,” facilitate better growth of cultures than standard cool/warm white light-emitting ones.
  13. Use low light intensity during culture initiation in order to prevent leaves turn red and slowing of shoot growth. The highest irradiance (even 100  $\mu\text{mol}/\text{m}^2/\text{s}$  PPFD) is recommended in the last week of subculture before shoot rooting.
  14. Usually shoots of *Vaccinium* sp. are recalcitrant to root in vitro contrary to ex vitro conditions. Rooting in vivo is also simple, cheap, and avoids a single step of tissue culture for rooting. Rooted microshoots of highbush blueberry resemble seedlings (see Fig. 2g, h)
  15. The young micropropagated nursery plants (see Fig. 2i, j) can be used as source of cuttings rooted conventionally. The solution of dikegulac (1 g/L) could be used to stimulate branching of highbush blueberry (29) (see Fig. 2k, l).
  16. No need to use auxins to rooting as shoots of many cultivars can root in their absence. In some cases auxin treatment results in the reduction of the number of rooted shoots, however sometimes has a positive influence on plant growth in the nursery.
  17. As there is evidence that mycorrhiza improve the field performance of shrubs and their drought and pH tolerance ((30), Personal information) consider application of fungi on young acclimatized plants.

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## In Vitro Propagation of Peanut (*Arachis hypogaea* L.) by Shoot Tip Culture

Elif Aylin Ozudogru, Ergun Kaya, and Maurizio Lambardi

### Abstract

Peanut (*Arachis hypogaea* L.), also known as groundnut, is the most important species of *Arachis* genus, originating from Brazil and Peru. Peanut seeds contain high seed oil, proteins, amino acids, and vitamin E, and are consumed worldwide as edible nut, peanut butter, or candy, and peanut oil extracted from the seeds. The meal remaining after oil extraction is also used for animal feed. However, its narrow germplasm base, together with susceptibility to diseases, pathogens, and weeds, decreases yield and seed quality and causes great economic losses annually. Hence, the optimization of efficient in vitro propagation procedures would be highly effective for peanut propagation, as it would raise yield and improve seed quality and flavor. Earlier reports on traditional micropropagation methods, based on axillary bud proliferation which guarantees the multiplication of true-to-type plants, are still limited. This chapter describes a micropropagation protocol to improve multiple shoot formation from shoot-tip explants by using AgNO<sub>3</sub> in combination with plant growth regulators.

**Key words:** *Arachis hypogaea* L, Micropropagation, Peanut, Shoot tips, Silver nitrate, 6-Benzyladenine,  $\alpha$ -Naphthaleneacetic acid.

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### 1. Introduction

Peanut (*Arachis hypogaea* L.), an annual grain legume crop, is by far the most economically important species of the genus *Arachis*. It has a special place among domesticated plant species, as it produces the seeds below the soil while having the flowers above the ground (1). Peanut is originated from Brazil and Peru (the earliest archaeological records from Peru date back to 3900–3750 bc), and believed to be distributed to Old World by Spanish and Portuguese explorers, probably loaded as a food supply to the ships carrying slaves from Africa to New World (2). Today, the plant is cultivated

worldwide in tropical, subtropical, and warm temperate climates (2), especially due to its very high nutritional value. Peanut seed, currently being the fourth most important oilseed worldwide (3), contains 36–54% oil and 20–25% protein, as well as amino acids (such as thiamine, niacin, and riboflavin) and vitamin E (4), and is either consumed directly as a food or is crushed for oil production. The meal remaining after oil extraction is an important source of animal feed, as well (1).

Peanut is propagated mostly by seed. However, as majority of the cultivation is in tropical or subtropical regions, seeds in those regions are usually conserved at high temperature and moisture conditions and, as a consequence, seed quality is often not sufficient for efficient propagation. The suborthodox storage behavior of the seeds, due to their high lipid content and fragile tegument which may lead to oxidative damages (5), also contributes to such difficulties of seed storage. High seed moisture at the time of harvesting leads to frost damages, especially in Northern countries, and to significant reduction in quality and flavor of seeds. In addition, due to the indeterminate growth habit of peanut, the harvested yield usually contains both mature and immature seeds, causing a significant economic loss due to the decline in seed weight and quality (1). Lack of moisture and calcium, pathogens, insects, and weeds damage the peanut pods, making them unfit for commerce, and restricting the propagation of peanut (1, 6).

In vitro propagation methods provide an important alternative for peanut mass propagation. Although the cultivated peanut is still known to be relatively recalcitrant to tissue culture (7), successful protocols of micropropagation (8–11), organogenesis (12–19), and somatic embryogenesis (20–33) have been reported from various explant types. This chapter describes a detailed micropropagation protocol developed in our laboratory (8).

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## 2. Materials

Tissue culture facilities: balance, analytical balance, magnetic stirrer with heating function, pH meter, refrigerators at 4 and  $-20^{\circ}\text{C}$ , autoclave, laminar air flow cabinet, glass bead sterilizer or spirit burner to sterilize the stainless steel instruments, stainless steel scalpels and forceps, electronic pipette,  $0.22\ \mu\text{M}$  syringe filters, syringes, climatic chamber, or culture room.

### 2.1. Seed Sterilization

1. 70% ethanol (EtOH).
2. 10% hydrogen peroxide ( $\text{H}_2\text{O}_2$ ).
3. 20% commercial bleach “Domestos” (at 2% active chlorine, Unilever, London, UK).

4. Sterile dH<sub>2</sub>O.
5. Sterile cylinders.
6. Sterile glass containers (jars), closed tightly by a cap.
7. Sterile beaker(s).
8. Sterile Whatman filter papers.
9. Sterile Petri dishes (Ø 9 cm).
10. Sterile cutting pads.

### **2.2. Preparation of the Culture Media**

1. Glass culture tubes (Sigma<sup>®</sup>, UK), Petri dishes, baby food jars (Sigma<sup>®</sup>, UK) and vitrovent boxes (Duchefa, The Netherlands).
2. Stock solutions for macroelement, microelement, vitamin, and organic elements of MS medium (34).
3. 1 N NaOH and 1 N HCl.
4. Sucrose.
5. Agar.
6. Stock solutions for 6-benzyladenine (BA),  $\alpha$ -naphthaleneacetic acid (NAA), and silver nitrate (AgNO<sub>3</sub>).
7. Beakers and cylinders.
8. Magnetic stir bars.
9. Stretch film or parafilm.

### **2.3. In Vitro Seed Germination**

1. Semisolid MS medium, devoid of plant growth regulators, supplemented with 3% sucrose (w/v) and 0.7% agar, contained in glass culture tubes (15 mL medium per tube), baby food jars (40 mL medium per jar), or vitrovent boxes (100 mL medium per box).
2. Stretch film or parafilm.

### **2.4. Explant Excision and In Vitro Culture**

1. Sterile cutting pads.
2. Semisolid MS medium, supplemented with 33  $\mu$ M BA, 5.3  $\mu$ M NAA, and 23.5  $\mu$ M AgNO<sub>3</sub>, 3% sucrose (w/v) and gelled with 0.7% agar (proliferation medium), contained in glass Petri dishes (Ø 9 cm).
3. Stretch film or parafilm.

### **2.5. In Vitro Propagation**

1. Sterile cutting pads.
2. Sterile baby food jars or vitrovent containers, containing 40 and 100 mL proliferation medium, respectively.
3. Sterile vitrovent containers, containing 100 mL of semisolid MS medium, devoid of growth regulators, supplemented with 3% sucrose (w/v) and gelled with 0.7% agar.
4. Stretch film or parafilm.

**2.6. Acclimatization of In Vitro Plantlets**

1. Tap water.
2. Plastic pots (0.5 l).
3. Autoclaved peat and perlite mixture (1:1).
4. Climatic chamber or greenhouse, providing 90% relative humidity and 25°C temperature.

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**3. Methods**

The micropropagation protocol described here is based on the use of AgNO<sub>3</sub> in the culture medium which, in combination with plant growth regulators, has a positive effect on multiple shoot formation of peanut through shoot tip culture (8).

**3.1. Preparation and Sterilization of Culture Media**

1. Prepare MS basal medium according to the formulations given in Table 1. Add required amount of stock solutions of macroelements, microelements, iron, vitamins, and organic elements in a beaker and stir the solution on a magnetic stirrer (see Note 1).
2. Increase the volume to about ½ of the final desired medium volume by adding dH<sub>2</sub>O.
3. Add 30 g/L sucrose and 100 mg/L myo-inositol to the medium and stir well until they are dissolved completely. When the powder compounds are dissolved completely, adjust the volume of the medium to the final desired volume by adding doubled distilled water and keep stirring for another minute.
4. For germination and rooting, use semisolid MS medium devoid of plant growth regulators. To induce shoot proliferation from excised shoot tips, add 33 µM BA, 5.3 µM NAA, and 23.5 µM AgNO<sub>3</sub> to the proliferation medium (see Notes 2 and 3)
5. Adjust the pH of the medium to 5.8 by adding 1 N NaOH or 1 N HCl.
6. Dispense the medium into Duran glass bottles (Sigma®, UK), containing 7 g/L agar, and autoclave at 121°C for 20 min (1.4 kg/cm<sup>2</sup>).
7. Dispense medium in different glasswares or plasticwares: (a) germination medium into glass culture tubes (15 mL), baby food jars (40 mL), or vitrovent boxes (100 mL); (b) proliferation medium into glass Petri dishes (Ø 9 cm), baby food jars (40 mL), or vitrovent boxes (100 mL); and (c) rooting medium into vitrovent boxes (100 mL) under laminar flow cabinet (see Note 4).

**Table 1**  
**MS basal medium formulation [34], used for the preparation**  
**of germination, multiplication, and rooting media**

Stock solutions	Preparation of the stock solutions (mg/L)	Preparation of MS media (mL/L) <sup>a</sup>
<i>Macroelement stocks (×20)</i>		
NH <sub>4</sub> NO <sub>3</sub>	1,650	20.6
KNO <sub>3</sub>	1,900	18.8
KH <sub>2</sub> PO <sub>4</sub>	170	5.0
CaCl <sub>2</sub> ×7H <sub>2</sub> O	440	12.0
MgSO <sub>4</sub> ×7H <sub>2</sub> O	370	6.0
<i>Microelement stock (×100)</i>		
H <sub>3</sub> BO <sub>3</sub>	6.20	10
KI	0.83	
MnSO <sub>4</sub> ×4H <sub>2</sub> O or MnSO <sub>4</sub> ×H <sub>2</sub> O	22.30	
ZnSO <sub>4</sub> ×7H <sub>2</sub> O	16.90	
Na <sub>2</sub> MoO <sub>4</sub> ×2H <sub>2</sub> O	8.60	
CuSO <sub>4</sub> ×5H <sub>2</sub> O	0.25	
CoCl <sub>2</sub> ×6H <sub>2</sub> O	0.025	
<i>Iron stock</i>		
FeSO <sub>4</sub> ×7H <sub>2</sub> O	27.8	10
Na <sub>2</sub> EDTA	40.0	
<i>Organic element stock</i>		
Glycine	2.0	10
Nicotinic acid	0.5	
Thiamine HCl	0.1	
Pyridoxine HCl	0.5	

<sup>a</sup>Note that for macroelements, stock solutions were prepared separately, while for microelements, iron and organic elements unique solutions containing a mixture were prepared

8. Keep the media under laminar flow cabinet until they are solidified completely.
9. Secure the caps of the containers by using parafilm or stretch film, label carefully the medium content in each container, and store them at 4°C in darkness.

### 3.2. Seed Sterilization and Germination In Vitro

1. Remove seed shells and isolate intact seeds (see Note 5, Fig. 1a).
2. Transfer them inside a sterile glass jar, containing 70% ethanol, and shake well for 5 min (see Notes 6 and 7, Fig. 1b).
3. Wash seeds with sterile dH<sub>2</sub>O for 5 min by shaking gently.



Fig. 1. Decontamination of peanut seeds. (a) Intact seeds, free of seed shells; (b) glass jars where to treat the seeds in the sequence of 70% EtOH,  $H_2O_2$ , and 20% commercial bleach solutions; (c) taking off the seed coats; (d) seeds, left to dry on filter papers.

4. Transfer seeds in a glass jar, containing 10%  $H_2O_2$ , and shake well for 5 min (see Note 8).
5. Wash seeds with sterile  $dH_2O$  for 5 min by shaking gently.
6. Transfer seeds in a glass jar, containing 20% commercial bleach “Domestos,” and shake well for 15 min (see Note 9).
7. Wash seeds with sterile  $dH_2O$ , at least for three times and 5 min each, until the bleach is completely discarded.
8. Keep remaining seeds in  $dH_2O$  and transfer seeds one by one on a sterile cutting pad to remove their seed coats by using sterile scalpels (see Fig. 1c).
9. Transfer seeds on a sterile filter paper in a Petri dish and let them dry at least for 30 min (see Fig. 1d).
10. Transfer dry seeds on semisolid MS medium devoid of plant growth regulators, contained in glass culture tubes, baby food jars, or vitrovent boxes (see Note 10).
11. Seal containers by parafilm or stretch film and incubate them in climatic chamber or culture room at  $25 \pm 1^\circ C$ , under 16 h photoperiod, provided by cool daylight fluorescent lamps ( $36 \mu mol/m^2/s$ ).

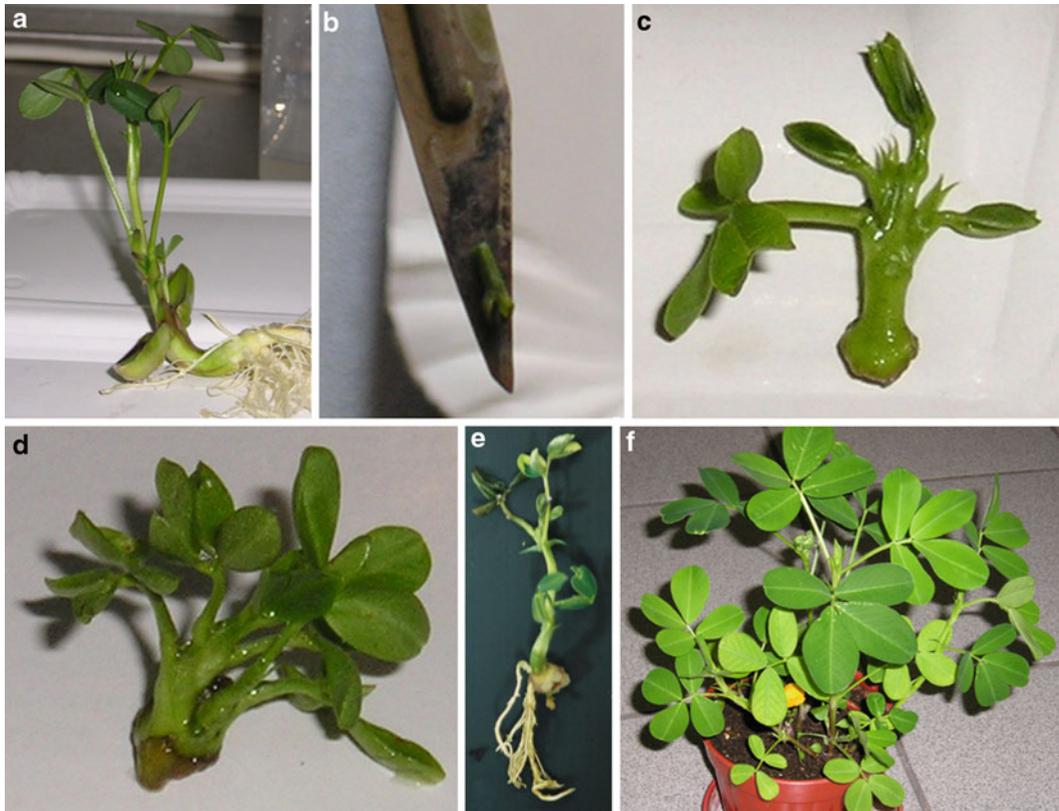


Fig. 2. In vitro propagation of peanut. (a) Germinated seedling, served as explant source; (b) shoot tip, excised from seedling and used for in vitro culture initiation; (c) shoot proliferation, 15 days after shoot tips were plated on medium; (d) multiple shoots, obtained at the end of first subculture period (4 weeks); (e) rooted shoot; (f) peanut plants in pot, obtained by in vitro multiplication, rooting, and acclimatization.

### 3.3. Establishment and Multiplication of In Vitro Shoot Cultures

1. After 2 weeks, excise shoot tips, 3–4 mm long, from well-germinated seedlings under laminar flow cabinet (see Note 11, Fig. 2a, b); transfer to Petri dishes containing semisolid MS medium supplemented with 33  $\mu\text{M}$  BA, 5.3  $\mu\text{M}$  NAA, and 23.5  $\mu\text{M}$   $\text{AgNO}_3$ .
2. Seal the Petri dishes by parafilm or stretch film and incubate them in climatic chamber or culture room at  $25 \pm 1^\circ\text{C}$ , under 16 h photoperiod, provided by cool day light fluorescent lamps ( $36 \mu\text{mol}/\text{m}^2/\text{s}$ ) (see Note 12).
3. Four weeks later, isolate single shoots from regenerated multiple shoot clusters and subculture them on the fresh medium of the same composition, contained in baby food jars or vit-rovent boxes (see Fig. 2c, d).
4. Seal the containers by parafilm or stretch film and incubate them in climatic chamber or culture room at  $25 \pm 1^\circ\text{C}$ , under 16 h photoperiod, provided by cool daylight fluorescent lamps

( $36 \mu\text{mol}/\text{m}^2/\text{s}$ ). Maintain the cultures by subculturing in 4-week intervals to fresh culture medium (see Note 13).

5. Check the cultures periodically, and discard immediately contaminated cultures. Autoclave contaminated glass or baby food jars before reuse.

### **3.4. Shoot Rooting and Acclimatization**

1. After at least three cycles of multiplication, isolate single shoots (minimum 20 mm) and transfer them on semisolid MS medium devoid of plant growth regulators, contained in vitrovent boxes.
2. Seal the containers by parafilm or stretch film and incubate them in climatic chamber or culture room at  $25 \pm 1^\circ\text{C}$ , under 16 h photoperiod, provided by cool daylight fluorescent lamps ( $36 \mu\text{mol}/\text{m}^2/\text{s}$ ). Shoots are rooted within 4 weeks (see Note 14, Fig. 2c).
3. Take out the rooted shoots from the containers and wash roots gently in running tap water in order to remove the sticking medium.
4. Transfer the plantlets into 0.5 L plastic pots, containing sterilized peat and perlite (1:1) mixture and place them in a climatic chamber at  $25 \pm 1^\circ\text{C}$ , under 16 h photoperiod, providing 90% relative humidity.
5. Transfer potted plants in reduced relative humidity condition gradually within 8 weeks (see Notes 15 and 16) and finally to the field (see Note 17, Fig. 2f).

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## **4. Notes**

1. Stock solutions of macro-, microelements, and iron are prepared according to the formulations given in Table 1 and stored at  $4^\circ\text{C}$  in transparent bottles; iron solution is stored in a dark bottle. Solutions with cloudy-like contaminants should be discarded immediately. Stock solution of organic elements is dispensed in plastic bottles, each bottle containing 20 mL solution, and stored at  $-20^\circ\text{C}$ .
2. Stock solutions of plant growth regulators are stored at  $4^\circ\text{C}$ .
3. BA and NAA are sterilized by autoclaving, thus they are included in the medium before sterilization.  $\text{AgNO}_3$  is a heat-sensitive compound, added to the autoclaved medium, under the laminar flow cabinet, by sterilization with  $0.22 \mu\text{M}$  syringe filters when the medium cools down to the temperatures of about  $40^\circ\text{C}$ .

4. Seed germination rate is better and faster in vitrovent boxes due to gas permeable nature of the containers. For germination, place one seed in each glass test tube, 2–4 seeds in baby food jars, and 7–9 seeds in vitrovent boxes. Excised shoot tips, derived from seedlings, are cultured on proliferation medium contained in Petri dishes. The selection of a container during multiplication and rooting is dependent on the scale of experiment.
5. Seeds with or without shells should be stored in tightly closed glass containers at 4°C in the darkness. Keep dry condition inside the container and check periodically for the presence of mould.
6. Ten min EtOH treatment was equally effective to seed sterilization, and thereby shorter treatment of 5 min is preferred.
7. Shaking the jars quite strongly by hand makes easier the removal of the seed coats and increases the decontamination success.
8. Five minute H<sub>2</sub>O<sub>2</sub> treatment was appropriate for seed germination. Use sterile plastic gloves while handling H<sub>2</sub>O<sub>2</sub>.
9. Domestos is a commercial bleach, containing 2% active chlorine. Alternatively, use similar concentration of NaOCl solution added with a few drops of Tween 20.
10. This seed decontamination protocol gave 99% sterilization rate.
11. Within 2 weeks, about 98% seed germination is obtained upon seed transfer to semisolid MS medium, devoid of plant growth regulators.
12. Shoot tips should start to elongate after 5–6 days. Multiple shoot formation begins after 15–20 days.
13. Addition of AgNO<sub>3</sub> (23.5 µM) in the proliferation medium has a positive impact on shoot tip proliferation, and results in 25% regeneration rate and 6.3 shoots per shoot tip. AgNO<sub>3</sub> shows a positive and marked effect on both shoot elongation and the reduction of callus proliferation from the basal ends of shoot tips, as well.
14. Long-term culture in AgNO<sub>3</sub>-containing media do not produce any residual effect on shoots during in vitro rooting on semisolid MS medium devoid of plant growth regulators; rooting gets easier and acclimatization is successful in the greenhouse conditions. Rooting rate of peanut microshoots is over 78%.
15. Alternatively, acclimatization can be done in the greenhouse, by covering the pots with a plastic cover. Relative humidity can be decreased gradually by making holes in the plastic cover in 3–4 day intervals.

16. Survival of the rooted shoots at the end of 8 weeks is about 90%, and they can be transferred to the normal growing conditions. Gynophores are obtained in about 1–2 months after blooming of the potted plants, and seeds about 2 months after that.
17. Following this protocol, after introduction to *in vitro* and the first 4 weeks of culture, a gradual increase in shoot tip regeneration frequency is obtained (from 24 to 35%, on average). Hence, about 5,600 rooted plantlets can be obtained for transfer to the field from 100 original shoot tip explants, after 6 monthly subcultures, rooting, and acclimatization.

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# Chapter 7

## In Vitro Propagation of Persimmon (*Diospyros kaki* Thunb.)

Edgardo Giordani, Mar Naval, and Carla Benelli

### Abstract

Persimmon (*Diospyros kaki* Thunb.) is a temperate fruit tree species diffused in all continents. The traditional propagation method adopted by the nursery industry is based on budding/grafting scion cultivars on seedlings from *D. kaki*, *Diospyros lotus*, and *Diospyros virginiana*, the most important species used as rootstock, reproduced by seeds since they are not easy to root. Furthermore, most of nonastringent cultivars of persimmon are not compatible with *D. lotus*, a rootstock largely utilized because of its hardiness and frost resistance. The main in vitro tissue culture techniques, developed for persimmon, deal with direct regeneration (from dormant buds and root tips), and indirect regeneration through callus from dormant buds, apices, and leaves. The bottlenecks of micropropagation are (1) the recalcitrance of many cultivars to in vitro establishment, (2) the low multiplication ratio of *D. kaki* compared to other fruit tree species, (3) the very low rooting ability of ex novo microcuttings both from direct and indirect regeneration, (4) the high sensitivity to transplant from in vitro to in vivo conditions. The development of reliable in vitro regeneration procedures is likely to play a key role for production of both clonal rootstocks and self-rooted cultivars. The general protocol for micropropagation of persimmon reported here is based on the establishment of winter dormant buds in vitro, shoot development, multiplication and elongation, and shoot rooting, using cytokinins (BA or zeatin) in a MS media along with an auxinic pre-treatment for rooting induction.

**Key words:** Astringency, *Diospyros* spp., In vitro culture, Recalcitrant, Rooting

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### 1. Introduction

The word “Persimmon” is generally attributed to the Oriental Persimmon (*Diospyros kaki* Thunb.), presumably originated in China, being *Diospyros lotus* L. and *Diospyros virginiana* L. two related species used as rootstock in many areas. Persimmon, a polygamous-dioic species, produces berries by parthenocarpy or after pollination; the fruits, with high nutraceutical value, can be astringent or nonastringent depending on the tannin solubility and

content at harvesting time (1). Persimmon is considered a low input demanding, hardy species; the trend of persimmon market is positive and world production reached over 3.8 million tons in 2009 (2), China being the most important producer of persimmons, followed by Korea, Japan, Brazil, Italy, and Spain. Persimmon propagation is hindered mainly by the low rooting ability of cuttings belonging to the species used as rootstock (namely, *D. kaki* for nonastringent cultivars, *D. lotus*, utilized in cold areas, and *D. virginiana* in heavy soils), being in vivo clonal multiplication of both rootstocks and self-rooted cultivars practically impossible (3). As a consequence, rootstocks consist of 1- to 2-year-old seedlings that are grafted/budded with the selected cultivar, hence the resulting persimmon orchards often show a wide variation in tree growth, usually associated to an undesirable high tree vigor. The attempts to clonally propagate root cuttings of suitable dwarfing rootstocks had poor results (3).

In vitro tissue culture could be a tool for enhancing its cultivation through massive propagation of rootstocks (e.g., direct regeneration from dormant buds, shoot tips, roots) and indirect organogenesis from callus obtained from meristematic tissue and leaves segments. Moreover, tissue culture is an important tool for improving breeding techniques (via somaclonal variation, mutagenesis, gene transfer, and production of haploids) and for germplasm conservation (e.g., by means of cryopreservation) (4). The initial research on persimmon tissue culture led to the formation of plantlets from callus cultures derived from immature embryos (5); later on, roots and initial buds were induced from cambial callus from adult trees (6). Direct regeneration from buds of single node sections was obtained by Cooper and Cohen (7), suggesting that massive in vitro micropropagation could be applied to persimmon. Soon after, the optimal conditions for shoot proliferation were investigated on young seedlings, mature embryos, and adult tissues. Differences among cultivars concerning shoot multiplication rate and rooting ability were observed (8). Key factors for the improvement of the protocols were to use modified MS (9) growing media and zeatin instead of other cytokinins. Nowadays persimmon, even if not commercially micropropagated, can be considered a quite advanced fruit tree species in tissue culture systems by means of shoot tip culture, regeneration from callus, endosperm, and protoplast (4, 10, 11); furthermore, regeneration protocols have been applied in gene transformation (12, 13). Several tissue culture techniques have been experimentally applied with success on Japanese cultivars (such as “Nishimurawase,” “Hiratanenashi,” “Gionbo”), as well as on Italian and Spanish cultivars (i.e., “Kaki Tipó” and “Rojo Brillante,” respectively). The Japanese “Jiro,” a nonastringent cultivar, is a model for in vitro tissue culture (Fig. 1). Nevertheless, in vitro massive micropropagation has never been applied for

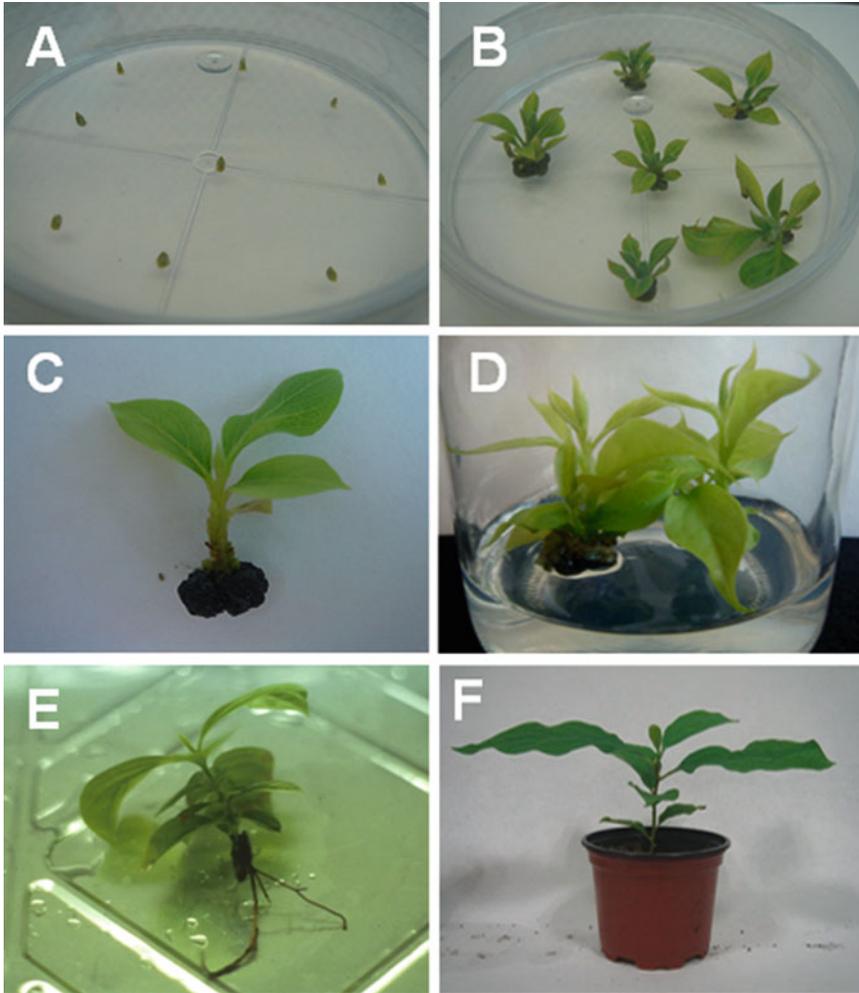


Fig. 1. In vitro propagation of persimmon. (a) Explants (axillary buds) of *D. kaki*, cv “Jiro,” cultured on modified MS medium, containing 5.0 mg/L BA. (b, c) Initial shoot development. (d) Shoot multiplication in modified MS medium, containing 4.4 mg/L zeatin. (e) Shoot rooting, after treatment with 50 % aqueous ethanol IBA solution (1.23 mM). (f) A potted micro-propagated plant of *D. kaki*, cv “Jiro”.

*D. kaki*, *D. lotus*, and *D. virginiana* [3]. The limiting factors are the recalcitrance to in vitro establishment for many *D. kaki* cultivars and *D. lotus* and *D. virginiana*, which is evidenced by media and tissue browning, low rooting ability of microcuttings, and low survival to transplant. In persimmon, gelling agents may contribute to overcome browning of media, and tissue, increase of shoot proliferation and growth rate (14). Explants from roots (instead of dormant buds) seemed to increase rooting ability of microcuttings (15). Recently, new techniques for improving rooting rate (over 90 %) of microcuttings have been identified (16). Micropropagation seems to induce reinvigoration (and not typical rejuvenation) on persimmon trees (17).

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## 2. Materials

### **2.1. Sterilization of Source Material**

1. Tap water.
2. Ethanol 70 % (v:v).
3. Commercial bleach solution (5 % (v/v) NaOCl), diluted 1:10 (v:v) with tap water.
4. Autoclaved distilled water.
5. Tween 20
6. Screw capped bottles (500 mL).
7. Magnetic bar.
8. 500 mL beaker (autoclaved).
9. Whatman No.1 filter paper.
10. Tissue culture facilities—instruments (scalpel, forceps, glass beads sterilizer), 4°C freezer, magnetic stirrer, binocular microscope, laminar flow bench, culture room.
11. Portions of 1-year-old shoots with dormant axillary buds, which are collected from in vivo grown trees as a source of explants.

### **2.2. Preparation of Culture Media**

1. Modified MS medium (with half  $\text{NH}_4\text{NO}_3$  and  $\text{KNO}_3$  concentrations) for (a) establishment of explants (axillary dormant buds), (b) shoot multiplication, (c) elongation, and (d) shoot rooting.
2. Bi-distilled water.
3. Beaker (1,000 mL).
4. 6-Benzyladenine (BA).
5. 6- $\gamma,\gamma$ -dimethylallylaminopurine (2-iP).
6. Zeatin.
7. Screw-capped Duran glass bottles, 500–1,000 mL capacity (Schott AG, Mainz, Germany).
8. Sterilized 3–9 cm diameter Petri-dishes (HYSIL, England).
9. Glass jars (250, 500 mL).
10. Tissue culture facilities: magnetic stirrer, magnetic bar, autoclave, balance: analytical balance, parafilm (American National Can, Greenwich, CT, USA).

### **2.3. In Vitro Propagation and Rooting of Shoots**

1. Autoclaved distilled water.
2. Whatman No.1 filter paper.
3. Screw capped bottles (500 mL).
4. Petri dishes.
5. Indole-3-butyric acid (IBA).

6. Tissue culture facilities: scalpel, forceps, glass beads sterilizer, binocular microscope, laminar flow bench, culture room.

#### **2.4. Acclimation of Plantlets to Ex Vitro Conditions**

1. Tap water.
2. Commercial plastic pots (9 cm diameter).
3. Potting media consisting of mixture of peat, vermiculite, and perlite (2:1:1 by volume).
4. Transparent plastic bags.
5. Tissue culture facilities: glasshouse, bench, mist, drip irrigation.

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### **3. Methods**

Micropropagation is based on shoot regeneration, multiplication, and elongation obtained after a variable number of subcultures of explants, initially derived from dormant axillary buds collected from in vivo growing trees. The portion of branches, from which axillary buds are excised, is collected during the winter after chilling requirement has been satisfied (January for the Mediterranean climate). This procedure involves the following three steps:

- *Step 1:* Selection of source material and establishment of axenic cultures of axillary bud explant. Axillary buds are established in culture medium after removal of their scales and outer leaves in order to avoid the development in vitro of fungal and bacterial infection (see Note 1).
- *Step 2:* Culture, multiplication, elongation, and maintenance of explants on a modified MS medium, added of cytokinins to promote bud development.
- *Step 3:* Induction of roots on regenerated shoots using pre-treatment with auxin. Rooted plantlets require then a period of adaptability to ex vitro conditions (acclimation).

#### **3.1. Plant Material and Explant Decontamination**

1. Wrap in plastic bags the shoots of the last season's growth, collected during winter, and store the bags at 4°C until use.
2. Surface sterilize the shoots with ethanol spray. Excise good axillary buds (typical shape, big size, noninjured) from the shoots.
3. Surface sterilize the buds by stirring for 5 min in a 2 % sodium chloride solution after adding 2 drops of Tween 20.
4. Wash the explants with sterile distillate water three times, each for 5 min, before blotting the explants dry on sterile Whatman No. 1 filter paper.
5. First, excise the bud from the shoot, cutting it at the base. Then, remove under a binocular microscope bud scales and outer leaf primordial. Use a sterile scalpel for all the operations.

### 3.2. Preparation and Sterilization of Culture Media

First prepare stock solution of growth regulators as follows:

1. *6-Benzyladenine (BA)*: weight 0.1 g BA and dissolve well with some drops of 1 N NaOH; add 100 mL distilled water: 1 mL of stock solution contains 1 mg of BA; then store the solution of growth regulators in the dark at 4°C. Stock solutions should be replaced every 2 weeks. BA may be sterilized together with the culture medium.
2. *Zeatin*: weight 0.01 g zeatin and dissolve well with some drops of 1 N NaOH; add 100 mL distilled water: 1 mL of stock solution contains 0.1 mg of zeatin. Store the stock solution in a conveniently labeled vial at 0°C. Sterilization by filtration is recommended.
3. *6-γ,γ-dimethylallylaminopurine (2-iP)*: weight 0.1 g 2-iP and dissolve well with some drops of 1 N NaOH; add 100 mL distilled water: 1 mL of stock solution contains 1 mg of 2-iP. Store the stock solution at 0°C. 2-iP may be sterilized together with the culture medium.

Then prepare culture media for (a) in vitro establishment of axillary buds, (b, c) shoot multiplication and elongation, and (d) root induction from regenerated shoots (Tables 1 and 2) or use a powdered medium sourced from a commercial supplier (see Note 2).

For bud development and shoot establishment, place the explants on modified MS basal medium, containing 20 g/L sucrose and 5 mg/L BA or 2.2 mg/L of zeatin (depending on the cultivars) and solidified with 2 % (w/v) of gellan gum (such as Gel-GRO) (see Note 3). As for shoot multiplication, use the same basal medium with 2.2 mg/L BA or 4.4 mg/L zeatin. For elongation replace BA or zeatin with 5 mg/L 2-iP. The medium for rooting is half-strength MS hormone-free, where the shoots are placed after dipping auxinic treatment (see Note 4).

1. Adjust the pH of the media to 5.8 using 1 M HCl or 1 M KOH.
2. Dispense the media into suitable containers, e.g., 300 mL into 500 mL capacity Duran bottles, or 100 into 500 mL glass jars.
3. Sterilize the culture media by autoclaving at 121°C for 20 min (118 Kpa steam pressure).
4. Medium in Duran bottles will be dispensed 10 or 25 mL on 3 or 9 cm diameter Petri dishes, respectively, for the introduction in vitro of axillary buds and initial shoot development.
5. Store the sterile culture media in darkness at 4°C for 1 month, at the latest.

**Table 1**  
**Modified MS medium, sucrose, and gelling agent content for the different phases of persimmon micropropagation (pH 5.8)**

Component	Concentration (mg/L)
<i>Macroelements</i>	
CaCl <sub>2</sub> × 2H <sub>2</sub> O	440
NH <sub>4</sub> NO <sub>3</sub>	825
KNO <sub>3</sub>	950
MgSO <sub>4</sub> × 7H <sub>2</sub> O	370
<i>Microelements</i>	
KI	0.83
CoCl <sub>2</sub> × 6H <sub>2</sub> O	0.025
KH <sub>2</sub> PO <sub>4</sub>	170
H <sub>3</sub> BO <sub>3</sub>	6.2
Na <sub>2</sub> MoO <sub>4</sub>	0.25
MnSO <sub>4</sub> × 4H <sub>2</sub> O	22.3
CuSO <sub>4</sub> × 5H <sub>2</sub> O	0.025
ZnSO <sub>4</sub> × 7H <sub>2</sub> O	8.6
FeSO <sub>4</sub> × 7H <sub>2</sub> O	27.8
Na <sub>2</sub> EDTA	37.3
<i>Vitamins</i>	
Glycine	2
Nicotinic acid	0.5
Pyridoxine HCl	0.5
Thiamine HCl	0.1
Myo-inositol	100
Sucrose	20000
Gelling agent <sup>a</sup>	2000

<sup>a</sup>(Gel-Grow-ICN®, Gerlite®, gellum gum)

**Table 2**  
**Growth regulators for different micropropagation phases of persimmon**

Phase	Growth regulator	Cultivar
Introduction in vitro and shoot establishment (MS ½ N)	BA 5.0 mg/L Zeatin 2.2 mg/L	Hiratanenashi, Jiro, Kaki Tipo, Fuyu
Shoot multiplication (MS ½ N)	BA 5.0 mg/L Zeatin 4.4 mg/L	Hiratanenashi, Jiro, Nishimurawase, Hana-gosho, Fuyu, Kaki Tipo
Shoot elongation (MS ½ N)	2-iP 5.0 mg/L	Hiratanenashi, Jiro, Nishimurawase
Shoot rooting ½ (MS ½ N)	50 % aqueous IBA (1.23 mM) ethanol solution	Hiratanenashi, Jiro, Kaki Tipo

### **3.3. Culture and Maintenance of Explants**

1. Place 1 or 6 bud explants on the medium in each 3 or 9 cm diameter Petri dish, respectively. Prepare a minimum of 100 explants per genotype. Seal the dishes with Nescofilm.
2. Incubate the explants at  $24 \pm 1^\circ\text{C}$  under a 16 h photoperiod, provided by cool-white fluorescent tubes (light intensity,  $60 \mu\text{mol}/\text{m}^2/\text{s}$ ).
3. After 28 days, small shoots (about 1.0 cm in height) should be formed from the cultured explants, with a mean of 2–4 shoots per explant.
4. Excise shoots and transfer them to the multiplication/elongation medium and incubate them under the conditions previously reported. After about 60 days of culture, regenerated/elongated shoots should reach approximately 2 cm in height with 3–4 leaves.
5. Repeat the subcultures and procedures in order to get the amount of shoots needed.
6. Evaluate the shoot regeneration capacity of cultured explants for each genotype (frequency of shoot induction and number of regenerated shoots per explant), as well as the average elongation of shoots.
7. Excise well-developed shoots 3–4 cm in height for rooting, leaving only two apical leaves.

### **3.4. In Vitro Shoot Rooting**

The most common rooting method is based on an auxinic pretreatment

1. Dip shoot base in a 1.23 mM indole-3-butyric acid (IBA) 50 % aqueous ethanol solution for 15–30 s (4).
2. After pretreatment, incubate the shoots in a hormone free growing medium at  $24 \pm 2^\circ\text{C}$  for 10 days in the dark and then for 20 days with a 16 h photoperiod ( $60 \mu\text{mol}/\text{m}^2/\text{s}$ ).

### **3.5. Acclimation of Rooted Shoots to Ex Vitro Conditions**

1. Remove the rooted shoots from the culture medium and wash the roots carefully with warm (approximately  $35^\circ\text{C}$ ) water to remove excess agar (see Note 5).
2. Transfer plants individually to 9 cm autoclaved diameter plastic pots, each containing a mixture of peat, vermiculite, and perlite (2:1:1 by volume).
3. Water the plants thoroughly.
4. Enclose the plants within their pots in transparent plastic bags. Maintain the regenerated plants in a growth room at  $20 \pm 2^\circ\text{C}$  with a 16 h photoperiod and  $60 \mu\text{mol}/\text{m}^2/\text{s}$  cool-white fluorescent light.
5. After 7 days, remove a corner from the top of each bag to reduce the relative humidity and to facilitate acclimation of the plants to ex vitro conditions.

6. Gradually open the top corners of the bags during 2 weeks; discard the bags after an additional 2 weeks.
7. Transfer the plants to a glasshouse in low light intensity, 24/20°C day/night temperature, high air humidity, good water supply conditions. Gradually expose to higher light intensity and to open field-like conditions; when the plants have reached a height of 40–50 cm they can be transplanted out in the field under shadow, then under direct sunlight.

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## 4. Notes

1. Even if bud scale removal is relevant in order to obtain axenic cultures, endogenous infectious fungi or bacteria have never been a major problem for culture establishment.
2. MS modified with half  $\text{NH}_4\text{NO}_3$  and  $\text{KNO}_3$  concentrations; Sigma-Aldrich Co. Ltd., Poole, UK.
3. Establishment, regeneration, elongation, and rooting of shoots are species- and, often, cultivar-specific. *D. lotus* and *D. virginiana*, as well as many persimmon cultivars, are very difficult, sometimes even recalcitrant, to in vitro propagation. The high amount of polyphenols in *D. kaki*, *D. lotus*, and *D. virginiana* tissues, including axillary buds, often causes browning and oxidation of media and cut tissues, strongly hindering in vitro establishment. It is worthy of note that, as for cv “Kaki Tipo,” the rate of well-established explants and subsequent elongation and maintenance of shoots is gelling-agent dependant, with Gel-GRO (ICN, Costa Mesa, CA) being more suitable for tissue culture than Difco Bacto Agar (14).
4. The most common rooting methods are based on an auxinic pretreatment, in cv “Kaki tipo” the shoots can be cultured in growing media enriched with IBA (1 mg/L) for 65 h in darkness and then transferred in hormone-free medium (16)
5. Prior to transfer of regenerated plants to compost, it is recommended to reduce roots to 2–3 cm in length and remove any dead or broken lateral roots.

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## Micropropagation of *Citrus* spp. by Organogenesis and Somatic Embryogenesis

Benedetta Chiancone and Maria Antonietta Germanà

### Abstract

*Citrus* spp., the largest fruit crops produced worldwide, are usually asexually propagated by cuttings or grafting onto seedling rootstocks. Most of *Citrus* genotypes are characterized by polyembryony due to the occurrence of adventive nucellar embryos, which lead to the production of true-to-type plants by seed germination. Tissue culture and micropropagation, in particular, are valuable alternatives to traditional propagation to obtain a high number of uniform and healthy plants in a short time and in a small space. Moreover, in vitro propagation provides a rapid system to multiply the progeny obtained by breeding programs, allows the use of monoembryonic and seedless genotypes as rootstocks, and it is very useful also for breeding and germplasm preservation. In this chapter, two protocols regarding organogenesis of a rootstock and somatic embryogenesis of a cultivar have been described.

**Key words:** Anther culture, Citrus, Epicotyl cuttings, Organogenesis, Somatic embryogenesis

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### 1. Introduction

*Citrus* spp., native of South East Asia and China, are cultivated in more than 100 countries, between approximately 40° N and 40° S around the world. According to the latest reports about world fruit crop production, *Citrus* are the ones more largely produced. Moreover, their production presents an increasing trend (from 118 Mt of 2007 to 124 Mt of 2009) (1).

The most commercially important *Citrus* species are oranges (*Citrus sinensis* L. Osbeck), tangerines (*Citrus unshiu* Marc., *Citrus nobilis* Lour., *Citrus deliciosa* Ten., *Citrus reticulata* Blanco and their hybrids), lemons (*Citrus limon* L. Burm. f.), limes (*Citrus aurantifolia* Christm. Swing.) and grapefruits (*Citrus paradisi* Macf.). *Fortunella*, *Poncirus*, *Microcitrus*, *Chymenia* and *Eremocitrus* are other genera of the family *Rutaceae*, related to *Citrus*.

The importance of *Citrus* spp. is linked to their economic value and to the nutraceutical proprieties of their fruits. Moreover, *Citrus* spp. are connected to the social background of the countries where they are grown, because many traditions, also those related to the cookery, imply the use of *Citrus* fruits. *Citrus* fruits are mostly eaten fresh, but a large part of the production, mainly of grapefruits and oranges, is also used for juice extraction. Furthermore, *Citrus* spp. are utilized in several fields, not only in food industry, such as the production of marmalades, candies, etc., but also, due to their richness in essential oils and polyphenols, in the cosmetic and pharmacy industries.

In addition, *Citrus* cultivation is going through a really favorable expansion period, thanks to the increasing demand of ornamental plants that leads to the intensification of nursery activity. This new frontier of *Citrus* industry is quite complex, with the possibility of offering to the market, mostly of North Europe, several genotypes (lemon, mandarin, kumquat, orange, etc.) and typologies of plants with different characteristics (with or without flowers or fruits, diverse size, and shape of canopy, etc.).

*Citrus* rootstocks are usually propagated by growing open-pollinated seeds, because most of them are highly polyembryonic, due to the occurrence of adventive nucellar embryos, which lead to the production of true-to-type plants by seed germination. With the aim of increasing the number and the quality of cultivars and rootstocks available to growers, biotechnology, tissue culture and micropropagation, in particular, offer to the nursery activity many practical advantages to speed up and to improve the vegetative propagation and the production of *Citrus* true-to-type and virus-free plants. Micropropagation, in fact, is a valuable alternative to traditional propagation to obtain a high number of uniform and healthy plants in a short time and in a small space. Moreover, in vitro propagation provides a rapid system, able to multiply the plant progeny obtained by breeding programs; in addition, hybrid seedlings may be evaluated as rootstocks, without waiting several years for flower and seed formation. Furthermore, micropropagation allows the use of monoembryonic genotypes as rootstocks (2) and it has also other useful possible applications, such as in the genetic transformation (3).

In the last decades, in vitro regeneration of several *Citrus* species has been performed using different media and explants, particularly: stem and epicotyl segments (4–13), transverse thin cell layer (TCL) from stem internodes (14), roots (8, 13, 15, 16), leaf sections (13, 16) and reproductive organs (17–20). So far, micropropagation protocols, efficient enough to reach the high multiplication rates requested by the nursery activity, are already available, but unfortunately, not for all *Citrus* genotypes. For this reason, it is necessary to set up improved protocols that allow the large-scale production of *Citrus* plants through in vitro morphogenesis, by either organogenesis or somatic embryogenesis.

In this chapter, two protocols regarding micropropagation through organogenesis of the rootstock Carrizo citrange and via somatic embryogenesis of the cultivar Avana mandarin have been reported.

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## **2. Organogenesis from Epicotyl Segments of Carrizo Citrange (*Citrus sinensis* (L.) Osbeck x *Poncirus trifoliata* L. Raf.)**

In vitro plant regeneration from epicotyl and internodal stem cuttings has been achieved for several *Citrus* genotypes, evaluating the influence of different factors, such as explant orientation, polarity and cut surface contact with the medium, treatments with different growth regulators, and distance of the epicotyl cuttings from the cotyledonary node, aimed to increase the efficiency of in vitro regeneration (7, 10, 12, 21, 22).

Troyer and Carrizo citranges (*Citrus sinensis* (L.) Osb. x *Poncirus trifoliata* (L.) Raf.) are the most widespread *Citrus* rootstocks, due to the productive and qualitative characteristics provided to the cultivar, to the polyembryony of their seeds and above all, due to their tolerance to the *Citrus Tristeza Virus* (CTV), that, since the first serious epidemic observed in Argentina in 1930, caused the death of millions of sweet orange trees grafted onto sour orange (*Citrus aurantium* L.) (23).

This protocol describes the in vitro shoot regeneration from epicotyl segments of Carrizo citrange. Seedlings are obtained by in vitro Carrizo citrange seed germination.

### **2.1. Materials**

#### **2.1.1. Seed Surface Sterilization and Culture**

1. Ripe Carrizo citrange fruits as seed source.
2. Filter paper.
3. 250 mL autoclaved beakers.
4. Ethyl alcohol 70% (v:v).
5. Commercial bleach solution, diluted 1:4 (v:v) with distilled water.
6. Sterilized distilled water.
7. Parafilm.
8. 100 mm diameter tissue culture Petri dishes.
9. Tissue culture facilities: laminar flow hood, forceps, scalpels, glass bead sterilizer, or burners.

#### **2.1.2. Culture Media**

1. Media used for seed germination (A), for shoot regeneration from epicotyl segments (B), and for root induction (C) are reported in Table 1.
2. Plastic/glass 1000 mL beakers.
3. 1000 mL graduated cylinders.
4. 1000 mL screw capped Pyrex bottle.

**Table 1**  
**Media composition for Carrizo citrange seed germination (A),  
 epicotyl segment organogenesis (B), and shoot rooting (C)**

Components	Media		
	A	B <sup>a</sup>	C <sup>b</sup>
MS Basal Salt Mixture	1×	1×	1/2×
MS Vitamin Mixture	1×	1×	1×
	mg/L		
Sucrose	30000	30000	30000
Ascorbic acid	500	–	–
Malt extract	500	–	–
FeNaEDTA	–	–	18.85
GA <sub>3</sub>	1	–	–
BAP	–	1	0.25
NAA	0.02	0.1	1.00
IBA	–	–	2.00
Agar	8500	8000	8500

<sup>a</sup>Growth regulators as reported by Bordón et al. (4)

<sup>b</sup>Growth regulators as reported by Al-Bahrany (43)

1×: concentration for 1 L

1/2×: half concentration for 1 L.

pH 5.8.

(BAP=6-benzylaminopurine, GA<sub>3</sub>=gibberellic acid; MS=Murashige and Skoog (42); NAA=α-naphthaleneacetic acid).

5. Petri dishes (100 mm diameter tissue culture dishes).
6. Tissue culture facilities: autoclave, pH meter, magnetic stirrers, spin bars.

### 2.1.3. Acclimatization of Plants to Greenhouse Conditions

1. Autoclaved Magenta Boxes (Sigma), squared plastic pots.
2. Autoclaved mix of peat moss and agriperlite (1:1).
3. Transparent plastic bags.

## 2.2. Methods

### 2.2.1. Culture Media Preparation and Sterilization

1. For a final volume of 1 L, fill a beaker with 500 mL of distilled water and add a magnetic stirrer. Put the beaker with the water on a mixer and start adding, in this order: salt and vitamin mixture, the carbon source, and finally the growth regulators (from 1 mg/mL stock solutions) using micropipettes. Adjust the pH of media to 5.8 with 1 N KOH or 1 N HCl, and then add distilled water till 1 L.

2. Put the agar in the bottle before adding the medium, without mixing. Leave the magnetic stirrer in the bottle, close it with the cap, and cover with silver paper.
3. Put the medium in the autoclave and sterilize at 110 kPa, 121°C for 20 min.
4. After that, when the temperature of the autoclave is lower than 60°C, extract the medium, mix it and put it under the laminar flow hood. In this sterile environment, pour the medium in Petri dishes. For seed germination and for the initiation of the culture, media are dispensed in Petri dishes (15 mL of medium per each dish). For rooting induction, instead, 20 mL of media are poured in glass test tubes, previously sterilized.
5. Leave Petri dishes cooling under the laminar flow hood, until the agar gelification.

#### 2.2.2. Seed Sterilization and *In Vitro* Culture

1. Extract seeds from fresh fruits and wash them under tap water to eliminate pulp residues (see Note 1).
2. After tegument removal, sterilize seeds under the laminar flow hood, putting them in a sterile beaker and immersing them for 5 min in 70% ethanol.
3. Eliminate the ethanol and add the 25% bleach solution.
4. After 20 min, drain the seeds and wash them with sterile distilled water for three times.
5. Using sterile forceps, place 8–10 seeds per each Petri dish, containing the germination medium (A) (see Table 1).
6. Seal Petri dishes with Parafilm (see Fig. 1a).
7. Incubate plates in the growth chamber at  $27 \pm 1^\circ\text{C}$  in the dark.
8. Observe the cultures weekly to individuate contaminations and to monitor the seed germination (see Fig. 1b).

#### 2.2.3. *In Vitro* Epicotyl Segment Culture

1. For the *in vitro* culture, select seedlings with at least 5 cm long epicotyl.
2. Eliminate, from seedlings, hypocotyl and cotyledons, then cut epicotyls in 1 cm-long segments and put them in culture horizontally (see Fig. 1c).
3. Put in each Petri dish ten epicotyl cuttings. Seal Petri dishes with parafilm.
4. Incubate plates in the growth chamber at  $27 \pm 1^\circ\text{C}$  under cool white fluorescent lamps (TMN 30 W/84, Philips, France), providing a photosynthetic photon flux density of  $35 \mu\text{mol}/\text{m}^2/\text{s}$  and a photoperiod of 16 h.
5. Observe weekly the culture. After 30 days of incubation, direct organogenesis with bud formation will be evident (see Fig. 1d); bud regeneration will be observed at both ends of the cuttings (see Fig. 1e).

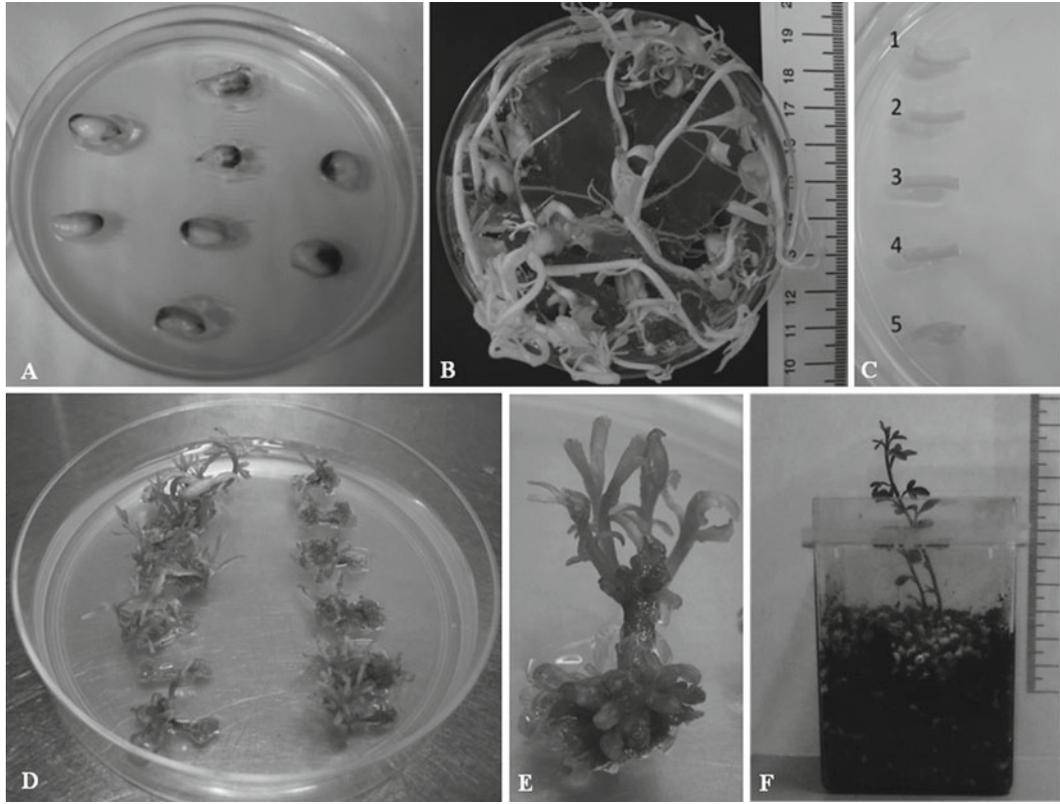


Fig. 1. Organogenesis from epicotyl segments of Carrizo citrange. (a, b) Carrizo citrange seed culture and germination. (c) Epicotyl segment culture; (d, e) Adventitious bud and shoot formation after 1 month of culture. (f) Rooted shoot transferred to in vivo conditions.

6. In almost 10 days, buds will develop into shoots. After 45 days of culture, shoots will be around 1 cm long.
7. Evaluate the regeneration efficiency recording, after 30 days of culture, the new adventitious bud production per segment (observing differences in the basal and apical part) and, after 45 days, the regeneration of shoots and their length.
8. Separate the shoots longer than 1 cm from the epicotyl cuttings, cut them in segments, about 5 mm long, containing at least one bud, put them in culture on the B medium, subculturing them every 45 days, on the same medium B (see Table 1).

#### 2.2.4. Root Induction of Regenerated Shoots

1. Select well-developed shoots and put them on the C medium (see Table 1), checking them weekly, until a good rooting system will be developed.
2. 40% of shoot rooting will be expected.

### 2.2.5. Acclimatization of Rooted Plants

1. Prepare a mix of peat moss and perlite 1:1 and wet the mixture with distilled water.
2. Fill for half content Magenta boxes (Sigma V8505) with the substrate and sterilize them in autoclave for 20 min at 121°C at 1 atm.
3. Extract rooted plants from the medium and carefully wash the roots with sterile distilled water.
4. Put the plantlets in the Magenta boxes with the perlite and peat moss mixture and store them in the growth chamber (see Fig. 1f).
5. After 2–3 weeks, if the plantlets exhibit a healthy foliar system, move them to the greenhouse covering the magenta boxes with plastic bags and regulating the humidity accumulation making some holes.
6. When the Magenta boxes will be too small for the plants, move them to a squared plastic pot, removing also the plastic bags.

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### 3. Somatic Embryogenesis via Anther Culture of *C. reticulata* Blanco, cv. Avana

Somatic embryogenesis, consisting in the differentiation of somatic cells into somatic embryos (bipolar structures without vascular connection with the original somatic tissue), plays an important role in many fields, particularly for large-scale vegetative propagation. This morphogenic process, that can occur with the formation of embryos emerging directly from the explants (direct somatic embryogenesis) or after the formation of callus (indirect somatic embryogenesis), has been reported in several species (19, 24). It is possible to induce somatic embryogenesis using different type of explants, culture media, and environmental conditions. The interest on somatic embryogenesis is due to several factors. In fact, thanks to the high regeneration efficiency and to the infrequent appearance of somaclonal variation (25), somatic embryogenesis has a key role in *in vitro* clonal propagation, for plant mass propagation (also using large-scale bioreactors), as well as for germplasm conservation and exchange, cryopreservation to establish gene banks, sanitation, metabolite production, and synthetic seed production. The application of synthetic seed technology to *Citrus* has been already studied, in order to set up efficient protocols for the encapsulation of somatic embryos of *Citrus reshni*, *Citrus reticulata* Blanco (cv. Avana and cv. Mandarin Tardivo di Ciaculli), *Citrus clementina* Hort. ex Tan. (cv. Monreal and cv. Nules), a lemon hybrid (26, 27) and Kinnow mandarin (28). Moreover, *in vitro* conservation of several *Citrus* species using encapsulation–dehydration technology of cryopreservation has also been reported (29, 30).

Anther culture is a commonly used method to produce haploids and doubled-haploids in *Citrus*, as well as in other fruit crops (31–33). However, it could also be employed to obtain somatic embryogenesis in many woody plants (18, 19). Regarding *Citrus*, anther culture produced somatic regenerants in *C. aurantium* (18, 34), *C. sinensis*, *C. aurantifolia* (35), *C. madurensis* (36), *C. reticulata* (18, 20), *Poncirus trifoliata*, the hybrid No. 14 of *C. ichangensis* x *C. reticulata* (37) and *C. paradisi* (unpublished).

In vitro anther culture is affected by numerous factors: genotype, pretreatment applied to anthers or to floral buds, pollen developmental stage, donor plant conditions, culture medium composition, and environmental growth conditions.

Here, the protocol to obtain embryogenic somatic callus by anther culture of a mandarin, cultivar Avana, has been reported.

### 3.1. Materials

#### 3.1.1. Floral Bud Surface Sterilization and Anther Culture

1. Immature *Citrus* flower buds with anthers at vacuolate stage.
2. See items 2–9 of Subheading 2.1.1 (60 mm diameter Petri dishes).
3. Acetocarmine staining solution 1%.
4. 1 mg/mL of 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI).
5. Stereo microscope, light microscope, fluorescent microscope.

#### 3.1.2. Culture Media

1. Media used for somatic embryogenesis induction (M1), embryogenic callus proliferation (M2), and embryo germination (M3) are reported in Table 2.
2. See items 2–6 of Subheading 2.1.2.

#### 3.1.3. Acclimatization of plants to greenhouse conditions

1. See items 1–3 of Subheading 2.1.3.

### 3.2. Methods

Depending on the season and on the genotype, *Citrus* floral buds are collected from plants growing in the field in the period of March–May. Flower buds must be collected at immature stage and stored at 4°C until the culture.

#### 3.2.1. Developmental Stage Determination

A correlation between flower size and pollen vacuolate stage (which is the most responsive for anther culture) was established. Only flower buds of this stage (see Fig. 2a), containing anthers 2.5–3.0 mm in length (see Fig. 2b) with microspores at the vacuolate stage are selected for culture, although different developmental stages have been observed within a single anther, and among different anthers of the same flower bud in *Citrus* and in *Poncirus* as well as in many other genera. The stage of pollen development is commonly determined by staining one or more anthers per floral bud size with acetocarmine (see Fig. 2c) or DAPI staining (see Fig. 2d).

**Table 2**  
**Media composition for mandarin anther culture**

Components	Media		
	Induction	Maintaining	Germination
	M1	M2	M3
NN Basal Salt Mixture	1×	–	–
MS Basal Salt Mixture	–	1×	1×
NN Vitamins	1×	–	–
MS Vitamins	–	1×	1×
Coconut water (v/v)	5%	–	–
		mg/L	
Casein	400	–	–
Glutamine	200	–	–
Sucrose	68000	50000	30000
NAA	0.2	0.02	0.01
Kinetin	1.0	–	–
BAP	0.5	–	–
ZEA	0.5	–	–
GA <sub>3</sub>	0.5	–	1.0
Agar	8000	8000	7500

pH 5.8

GA<sub>3</sub>, gibberellic acid; MS, Murashige and Skoog (42); NN, Nitsch and Nitsch (44); NAA,  $\alpha$ -naphthaleneacetic acid; BAP, 6-benzylaminopurine; ZEA, zeatin

### 3.2.2. Acetocarmine Staining

1. Squash anthers in 1% acetocarmine staining solution and observe slides under an optical microscope to determine the stage of pollen development.

### 3.2.3. DAPI staining

1. Squash anthers in few drops of DAPI solution (1 mg/mL) and observe slides under a fluorescent microscope to identify the pollen development stage.

### 3.2.4. Culture Media Preparation and Sterilization

1. See Subheading 2.1.1.

### 3.2.5. Flower Bud Sterilization

1. Sterilize flower buds, pretreated in the dark at 4°C for 10–15 days, in the laminar flow hood by immersion for 3 min in 70% (v/v) ethyl alcohol, followed by immersion in commercial

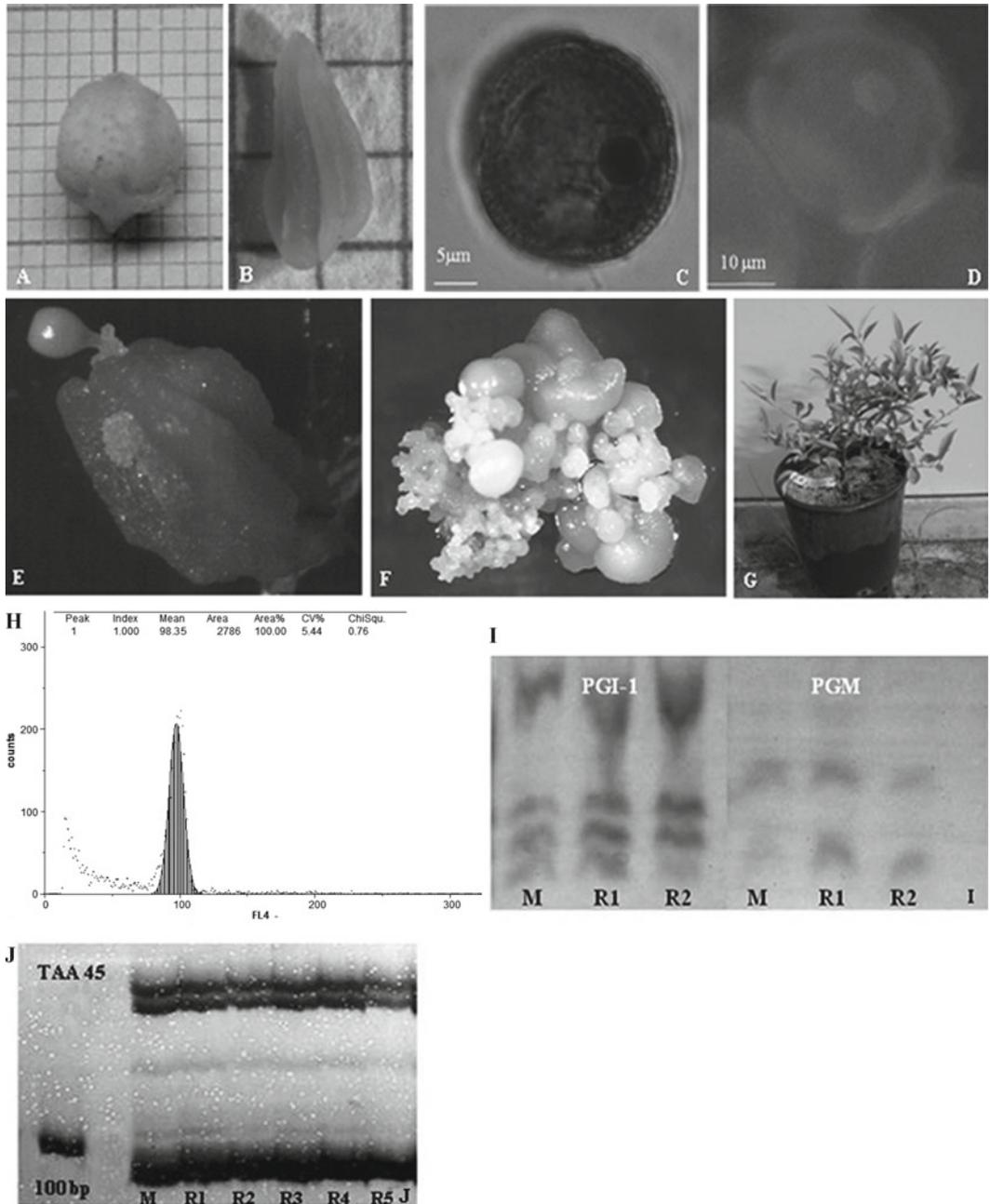


Fig. 2. Somatic embryogenesis via anther culture of *C. reticulata* Blanco, cv. Avana. (A) Flower bud (6 mm) of Avana mandarin containing anthers with microspores at the uninucleate stage. (B) Anther of mandarin Avana containing microspores at the vacuolate stage before culture. (C) Microspore at the vacuolate stage (acetic-carmin staining). (D) Microspore at the vacuolate stage (DAPI staining). (E) Callus and embryo production from an anther of Avana mandarin. (F) Morphogenic, friable callus, and cluster of embryos in different stages of development after 3 months of culture. (G) Plant of Avana mandarin regenerated from anther culture and transferred to ex vitro conditions. (H) Flow cytometer analysis: histogram of fluorescence intensity of mother plant leaf tissue and anther culture regenerant of *Citrus reticulata* Blanco. (I) Isozyme patterns of PGI (left) and PGM (right) of mandarin leaves: the first and the fourth lanes from the left are the zymograms of the mother plant (M), the other ones are those of the regenerants (R1–R2). (J) Polyacrylamide gel electrophoresis of microsatellites TAA45 showing the heterozygosity of regenerants from anther culture. DNA was extracted from leaves of mother plants (M) and of five anther culture regenerants (lanes R1–R5).

bleach solution (about 1.5% active chlorine in water) containing a few drops of Tween 20 for 15–20 min.

2. Finally rinse them three times for 5 min each, with sterile distilled water.

### 3.2.6. Anther Isolation and Culture

1. Remove petals aseptically with forceps.
2. Dissect anthers carefully, separating them from stamens.
3. Per each 60 mm-Petri dish containing 10 mL of solid medium, place 60–80 anthers onto the induction medium (M1) (see Table 2).
4. Seal Petri dishes with Parafilm, then incubate them at  $27 \pm 1^\circ\text{C}$  in the dark for 1 month, and then place under cool white fluorescent lamps (Philips TLM 30 W/84), with a photosynthetic photon flux density of  $35 \mu\text{mol}/\text{m}^2/\text{s}$  and a 16 h photoperiod.
5. Observe the cultures for 10 months, every 2 weeks.

### 3.2.7. Embryogenic Callus Maintenance

1. Anthers need 2–3 months to initiate callus and embryo production (see Fig. 2e).
2. Transfer embryogenic callus to the multiplication medium (M2) (see Table 2) to increase its amount (see Note 2).
3. Subculture the stock culture lines every 45 days, keeping them at the same light and temperature conditions.

### 3.2.8. Embryo Germination

1. Isolate and culture obtained embryos in the germination medium (M3) (see Table 2), in the light at  $27 \pm 1^\circ\text{C}$  (with a 16-h photoperiod) (see Note 3).
2. Culture embryos first in Petri dishes, then, after germination, transfer plantlets in Magenta boxes (Sigma V8505) or in test tubes containing the same medium, with 5–6 weeks of subcultures (see Note 4).

### 3.2.9. Plant Development and Acclimatization

1. See Subheading 2.2.5 (see Fig. 2g).

## 3.3. Regenerant Characterization

### 3.3.1. Ploidy Analysis of Regenerants

By anther culture, it is possible to obtain regenerants with different levels of ploidy. In fact, non-haploids may arise from somatic tissue of anther walls, from the fusion of nuclei, from endomitosis within the pollen grain, and from irregular microspores formed by meiotic irregularities. In *Citrus* and its relatives, the regeneration via anther culture of haploid, diploid, triploid, tetraploid, aneuploid, and mixoploid calli and plantlets have been reported (27, 38). Chromosome counts carried out on root apices of embryos and plantlets obtained from *in vitro* anther culture of mandarin, sour orange, and grapefruit showed the diploid set of chromosomes ( $2n = 2x = 18$ ) (18–20).

*3.3.2. Cytological  
Characterization*

Among the more used methods for the measurement of regenerant ploidy level, there is the chromosome number counting in root tip cells, using the standard Feulgen technique (39).

Material

1. Regenerated embryos.
2. 3:1, v/v ethanol: glacial acetic acid; EtOH; 1 N HCl.
3. Refrigerator.
4. Schiff's reagent.
5. Light microscope.

Method

1. Pretreat somatic embryos with 0.05% colchicine for 2 h at room temperature.
2. Fix material in 3:1, v/v ethanol: glacial acetic acid overnight. Store fixed samples in EtOH at 4°C until viewing.
3. Macerate tissue sample in 1 N HCl for 5–10 min at 60°C.
4. After the maceration, stain with Schiff's reagent.
5. Observe samples under a light microscope.

*3.3.3. Flow Cytometer  
Analysis*

Material

1. Razor blade.
2. Samples of the regenerants and young leaves of the mother plant.
3. Partec CyStain® UV Precise; Partec CellTrics®.
4. Partec (Münster, Germany) flow cytometer.

Method

1. To release the nuclei from the cells, chop by a razor blade 0.5 cm<sup>2</sup> samples of a regenerant line mixed with a young leaf of the mother plant in 1 mL of extraction buffer (Partec CyStain® UV Precise).
2. Filter the suspension through 30 µm nylon gauze filter (Partec CellTrics®) to remove debris.
3. Add the staining buffer (Partec CyStain® UV Precise) to the suspension.

Determine relative DNA content of the samples by a Partec (Münster, Germany) flow cytometer. The presence of only one peak on the DNA histogram indicates the diploid status of regenerants (see Fig. 2h).

*3.3.4. Biochemical and  
Molecular Characterization*

Regenerants obtained from anther culture could be heterozygous or homozygous, depending on their origin; in fact, they can

originate from a gametic or a somatic cell. To discriminate somatic regenerants, biochemical, such as isozymes, and molecular markers characterized by codominance, such as microsatellites, are reliable tools.

#### Isozyme Analyses

In *Citrus*, isozymes are widely used to identify the origin of calluses and plantlets obtained from anther culture (18, 20). In fact, isozyme techniques allow to distinguish between pollen-derived and somatic tissue when the enzyme is heterozygotic in the diploid condition of the donor plant and the regenerants are missing an allele or not. The crude extracts of embryos and plantlets obtained are analyzed using two enzyme systems: phosphoglucosomerase (PGI) and phosphoglucosomutase (PGM). Numbering for isozymes (PGI-1) and lettering for different allozymes are the same as used by Torres (40). *C. reticulata* is heterozygous for PGI-1 and PGM. According to Torres (40), the heterozygous mandarin parent is FI (F=allele specifies fast migration toward the anode enzyme; I=intermediate) in PGM, and WS (W=allele which specifies an enzyme migrating faster than F; S=allele specifies a slowly migrating enzyme) in PGI. For analysis of calli and leaves obtained from anther culture, the presence of two bands in PGM and three bands in PGI-1 can be regarded as heterozygous state (see Fig. 2i).

#### Materials

1. Young, tender, fast growing leaves of the mother plants and embryogenic callus or leaves regenerated from anther culture.
2. Distilled water.
3. L-Histidine buffer.
4. Tris-HCl 1 M.
5. Hydrolyzed starch.
6. Glycerol.
7. Suction and volumetric flasks.
8. Laboratory facilities: vacuum apparatus, refrigerator, freezer (-10 to -20°C), power supplies: BioRad Model 3000xi Computer Controlled Power Supply, gel apparatus, hot plate, pH meter, balances.

#### Methods

##### *Stock solution preparation*

##### L-Histidine buffer:

1. Dissolve in distilled water 20186 g/L L-histidine and 8.25 g/L citric acid.
2. Bring to volume with distilled water.

*Tris-HCl 1 M*

1. Dissolve in distilled water 121 g/L Trizma base.
2. Adjust the pH to 8 using HCl.
3. Bring to volume with distilled water.

*Gel preparation*

1. In a suction flask, prepare a solution containing 16 ml of Histidine buffer and 40 g of Hydrolized Starch, then bring the volume to 400 ml with distilled water.
2. Heat the solution to boiling, removing the excess gases applying vacuum.
3. Pour the gel into the gel tray, covering it with a glass plate to uniform its thickness.
4. Cool the gel overnight.

*Gel loading and extraction*

1. Remove glass plate and trim the gel 4 cm from the cathodal end.
2. Macerate a small amount of tissue (~100 mg) directly into a little square filter paper (3MM Whatman chromatography), then insert them in the gel.
3. Insert a plastic straw at the anodal and cathodal end of the gel.
4. Cover the gel with inert material and put the gel tray in contact with the L-Histidine buffer.
5. Regulate electric field conditions: Costant mA 45; Time: 3 h.

*Gel slicing*

1. Slice the gel using a taut, fine stainless or spring steel wire.
2. Move the wire slowly through the gel.
3. Place the slices in separate stains.

*Gel staining*

1. Immerse one gel slice per each staining solution (see Table 3).
2. Incubate the gel slices in the dark at 25°C at least for 2 h.
3. Stop reaction and fix in 10% glycerol.

**Microsatellite Analyses**

Microsatellite analysis is an alternative to isozymes to investigate the genetic nature of the anther culture regenerants. Microsatellites, short tandemly repeat DNA sequences, are suitable markers for several applications in genetic analyses because of their positive features as co-dominant inheritance and high polymorphism. In *Citrus*, Kijas et al. (41) described several microsatellites, such as TAA 1, TAA 15, TAA 27, TAA 33, TAA 41, TAA 45, TAA 52, CAGG 9 and CAC 23. To separate the regenerants with a somatic origin from the gametic ones, it is necessary to individuate among these microsatellites, which ones are heterozygous in the mother

**Table 3**  
**Staining solutions composition for isozyme analysis**  
**(PGI-1: Glucose-6-phosphate isomerase; PGM:**  
**Phosphoglucomutase)**

Components	Staining solutions	
	PGI-1	PGM1
Tris HCl 1 M pH 8	5 mL	5 mL
MgCl <sub>2</sub> ·6H <sub>2</sub> O	50 mg	50 mg
Fructose-6-P	50 mg	–
NADP <sup>+</sup>	5 mg	10 mg
Glucose-1-P		250 mg
MTT	10 mg	20 mg
PMS	2 mg	2 mg
Glucose-6-P DH <sup>a</sup>	20 units	20 units
Distilled water	up 50 mL	up 50 mL

<sup>a</sup>Add just before the incubation.

plant. The presence of two bands both in the regenerant and in the mother plant is considered the confirmation of the somatic origin of the regenerant (see Fig. 2j).

#### *DNA Extraction and Amplification*

##### Materials

1. Sterile eppendorf tubes.
2. Sterile 100 and 1000  $\mu$ L tips.
3. 1–20, 20–200, 100, and 1000  $\mu$ L micropipettes.
4. Gloves.
5. Sterile pestles.
6. Ethyl alcohol.
7. Liquid nitrogen.
8. Phenol.
9. Ammonium acetate.
10. Isopropanol.
11. Extraction buffer (EB) (stocks 100 mM Tris–HCl pH 8.0, 50 mM Na<sub>2</sub>EDTA pH 8.0, 500 mM NaCl, 10 mM  $\beta$ -mercaptoethanol, 3% sodium dodecyl sulfate, SDS).
12. TE buffer (10 mL of 1 M Tris–HCl (pH 8.0), 2 mL EDTA (0.5 M), Milli-Q water to 1000 mL).
13. Crushed ice.

14. Primers (such as TAA1, TAA15, TAA41, TAA 45 (41)).
15. dNTPs.
16. Template DNA.
17. Taq DNA polymerase (Amersham Biosciences, USA).
18. 10× PCR buffer (500 mM KCl, 15 mM  $\text{MgCl}_2$ , 100 mM Tris-HCl, pH 8.3).
19. Laboratory facilities: centrifuge, vortex, timer, liquid nitrogen container, biosafety cabinet, water bath, PT 100 thermal cycler (MJ Research, USA).

#### Methods

1. Prepare, per each sample, around 7 leaf disks or 150 mg of callus.
2. Under sterile biosafety cabinet, switch on the 37°C water bath and warm up the EB.
3. Switch on the centrifuge and cool it to 4°C.
4. Add liquid nitrogen to the eppendorf tube with the sample.
5. Using the sterile pestle, squeeze the sample to reduce it to powder.
6. Add 700  $\mu\text{L}$  of EB and vortex.
7. Incubate at 65°C for 10 min.
8. Centrifuge for 5 min at 13000 rpm.
9. Transfer the supernatant in a clean eppendorf tube, add 700  $\mu\text{L}$  of phenol and vortex for few seconds.
10. Centrifuge for 5 min at 13000 rpm, transfer the supernatant in a clean eppendorf tube.
11. Add 65  $\mu\text{L}$  of ammonium acetate ( $\text{NH}_4^+ \text{Ac}^-$ ), 450  $\mu\text{L}$  of cold isopropanol and mix lightly.
12. Centrifuge for 10 min at 13000 rpm, eliminate the supernatant and add 700  $\mu\text{L}$  of 70–75% of cold ethyl alcohol.
13. Centrifuge for 10 min at 13000 rpm, eliminate the ethyl alcohol, using a vacuum centrifuge, leaving uncovered the eppendorf tubes.
14. Resuspend DNA in 100  $\mu\text{L}$  of TE buffer.
15. Store at 4°C for one night.
16. Quantify or store at -20°C.
17. Working on ice and being careful about DNA contaminations, add in a 0.5 mL eppendorf tube all the reagents in the following order: 30  $\mu\text{L}$  sterile distilled  $\text{H}_2\text{O}$ , 5  $\mu\text{L}$  10× PCR buffer, 4  $\mu\text{L}$  dNTP Mix (1.25 mM), 2.5  $\mu\text{L}$  of each primer, 4  $\mu\text{L}$   $\text{MgCl}_2$  (25 mM) (see Note 5).
18. Just prior to starting the reaction add the Taq DNA polymerase (see Note 6).

19. Add 15  $\mu\text{L}$  of reagent mixture to the genomic DNA.
20. Place the eppendorf tubes in the thermocycler. Use the PCR thermal profile: 94°C for 5' for 1 cycle; 94°C for 60", 55°C for 30", 72°C for 60" for 32 cycles, 72°C for 5' (38).

### *Polyacrylamide (PA) Gel Electrophoresis*

#### Material

1. PA mixture: 6% acrylamide solution, 50  $\mu\text{L}$  N,N,N',N'-tetramethylethylenediamine (TEMED), 600  $\mu\text{L}$  10% ammonium persulphate (APS).
2. Gloves.
3. Laboratory facilities: polyacrylamide gel electrophoresis system.

#### Methods

1. Place 0.4 mm spacers on glass plates.
2. Pour the acrylamide mixture between the plates using a syringe (see Note 7).
3. When acrylamide solution fills the space between the plates, lay the plates flat.
4. Insert comb teeth up and clamp, then leave it to polymerize for 30–45 min.
5. Put the plates in the apparatus. Add in the chambers warm 0.5 X TBE ( $\pm 1$  cm over the shorter glass) (see Note 8).
6. Pre-run the gel at low wattage for 10 min (40°C).
7. Load the samples and connect the apparatus: 40°C, 40 W constant  $\pm 2$  h.
8. Stop the running, open the circuit, and eliminate TBE from upper chamber.
9. Open the glass plates, remove the spacers, and stain gel with silver staining.

### *Silver Staining*

#### Material

1. Polyacrilamide gel, tray.
2. Fixer (10% acetic acid): 50 mL glacial acetic acid in 450 mL distilled water.
3. Silver stain: 3 mL 1 N silver nitrate solution; 500 mL distilled water; sodium thiosulphate solution (0.1 N), formamide.
4. Developer: 15 g sodium carbonate; 500 mL distilled water and put it at 4°C.

#### Methods

1. Put the gel in a tray containing the fixer and leave it for 30 min.
2. Eliminate the fixer and wash two times the gel (15 min each).

3. Immerse the gel in the silver staining for 30 min (see Note 9).
4. Just before the gel developing, add 75 mL of sodium thiosulphate solution (0.1 N) and 0.75 mL of formamide to the cold developer solution.
5. Agitate the silver stain for 10 s before to eliminate it and then add the developer.
6. Wait the band development, than add the fixer.
7. Wash the gel with water for 20 min, then put it vertically to dry.
8. Photograph or scan the gel for observations.

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#### 4. Notes

1. Dry seeds on a filter paper and store at 4°C until use.
2. The average frequency of callus induction in *Citrus* is about 19% for mandarin, 59% for sour orange, 70% for grapefruit. Many research reported that most of the calli obtained in *Citrus* anther culture are non-morphogenic, but some of them appear friable, white, and differentiate into a clump of embryos (see Fig. 2f). This type of callus is highly embryogenic and its embryogenic potential is maintained for many years. From only one anther it is possible to obtain a high amount of embryogenic callus and more than 100 embryos after several subcultures.
3. In this medium, the somatic embryos will develop following the same steps of the zygotic ones: globular, heart, torpedo, and cotyledonary stages. Furthermore, secondary embryogenesis (direct and indirect) could be observed, more frequently in the root region of the embryos. Sometimes, teratomatal structures and morphological anomalies, cotyledonary-fused, pluricotyledonary or fasciated, and thickened embryos are observed (43).
4. A high conversion rate, around 80–89%, has to be expected; in fact, somatic embryos vigorously germinate *in vitro*.
5. The dose of each component has to be multiplied for the number of samples plus 1.
6. Taq DNA polymerase has to be kept on ice.
7. Inverting the syringe to expel any trapped air avoids bubble formation in the gel.
8. Warming the TBE reduces the time needed to bring the gel to the appropriate run temperature, before sample loading and preelectrophoresis run time.
9. The silver staining can be used up to ten times.

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## Micropropagation of *Prunus* Species Relevant to Cherry Fruit Production

Philippe Druart

### Abstract

Cherry tree micropropagation is limited to the production of healthy cultivars of *Prunus avium* and *Prunus cerasus*, and their rootstocks; mainly the dwarfing ones. By using meristem-tip (0.1 mm long) or healthy shoot tips/nodes, four successive steps are needed to obtain whole plants capable of growing in the nursery: multiplication by axillary branching, shoot elongation, rooting, and plantlet acclimation. Along this process, several parameters have to be adjusted for each phase of the culture, including media composition, environmental culture conditions and plant handling. These parameters vary depending on genotypic response and specific vulnerability to physiological disorders such as hyperhydricity, apex necrosis, unstable propagation, and rooting rates. Based on a 40 year-long experience of study and application of culture conditions to large-scale plant production, this document summarizes the main problems (variability of the propagation rate, hyperhydricity, apex necrosis, plant re-growth) and solutions encountered to solve them, with means validated on many mericlones.

**Key words:** Cherry tree, In vitro production, Management of micropropagation, Meristem culture, Micropropagation, *Prunus sp.*, Rooting

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### 1. Introduction

Cherry fruit species are part of the global benchmark; the world production was 2.3 million metric tons in 2009. The fruits of *Prunus cerasus* are sour and those of *Prunus avium* are sweet. During the last decade, there was a general tendency to increase the sour cherry cultivation area. The market demand of sour cherry fruits, mainly processed, is due to their curative, dietetic, and gustatory characteristics. The forecast for the world sweet cherry production is a 40% increase for the next 5 years. Europe, Turkey, United States, and Chile are amongst the most important regions of production (1–3).

*P. cerasus* can be grown on their own roots. The tree size is not excessive, enabling easy training operations and fruit harvesting. On the other hand, the cultivars of *P. avium* must be grafted onto rootstocks. These are seedlings from wild types of *P. avium* or clonal selections of *P. avium* or *Prunus mahaleb* propagated by cuttings and layering. However, these trees become out of control after a few years growth in the orchard. For the current intensive conditions of fruit production, dwarfing rootstocks have been selected among various ornamental *Prunus* species such as *Prunus serrulata*, *Prunus yedoensis*, or *Prunus subhirtella*, as well as among *Prunus cerasus* progenies.

Micropropagation of cherry trees therefore includes virus elimination in *P. avium* and the propagation of healthy rootstocks and *P. cerasus* cultivars.

In our laboratory, *Prunus* species have been propagated in vitro from meristem-tips culture (4) over the past 40 years. Amongst cherry-relevant species, several have been propagated at a commercial level, namely *Prunus dawyckensis* (GM 61) “Damil,” *P. canescens* (GM 79) “Camil,” *P. incisa* × *serrula* (GM9) “Inmil” that are dwarfing rootstocks used for sweet cherry orchards (5).

This chapter describes the in vitro culture conditions used for the propagation of fruit tree species relevant to cherry production, namely varieties and rootstocks.

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## 2. Materials

### 2.1. Surface Sterilization of Source Plant Material

#### 2.1.1. Meristem-Tip Culture

1. Reverse-osmosis purified water.
2. Absolute ethanol for disinfection procedures.
3. Calcium hypochlorite (9%).
4. Magnetic bars.
5. Magnetic stirrer.
6. 500 mL beakers.

#### 2.1.2. Shoot Tip and Node Culture

1. Formaldehyde solution (37%) for the disinfection of actively growing shoots.

### 2.2. Tissue Culture Facilities (see Note 1)

1. Medium cooking equipment.
2. Autoclave.
3. Instruments (watch chuck, razor blades, scalpels, forceps, and spirit burner to flame and sterilize tools).
4. Laminar flow bench.
5. Culture room with controlled temperature ( $23 \pm 1^\circ\text{C}$ ) and photoperiod (16 h) provided by Philips daylight TL 33 or

Sylvania gro lux (400–700 nm) lighting ( $50 \pm 7 \mu\text{E}/\text{m}^2/\text{s}$  at the plant level).

6. Cold rooms (+2, +4°C) and freezer to store plant material, stock solutions, and culture media components.
7. Thermo-hygrographs to record the temperature in the culture and storage rooms.
8. Stereo microscope for meristem-tip excision and plant material observations.
9. Kimax® (Kimble Chase article, Vineland) reusable borosilicate glass tubes (75–150 × 16 mm) with screw caps for meristem-tip culture.
10. “Sigma” culture tubes (165 × 25 mm) closed by kaputs for establishment of shoot tips/nodes and for first axillary branching cultures.
11. Reusable containers commonly used for plant sterilization (500 mL volume capacity) like former “Le Parfait” or “Weck” glass bottles types for multiplication, elongation, and rooting stages: the closure is a lid covered with cellophane sheet.
12. Transparent plastic boxes for transport and storage of in vitro plantlets before acclimation.

### 2.3. Culture Media

All the formulations of the macroelements, microelements, and vitamins used in the micropropagation protocols are presented in Table 1. Stock solutions are stored in the dark at 4–7°C for ±2 months.

#### 2.3.1. Meristem-Tip Culture Medium

Half-strength Murashige and Skoog’s macro elements (6), Nitsch’s microelements (7) with  $10^{-1}$  mM Fe ( $\text{FeNa}_2$  EDTA), vitamins of Jacquiot (8), 1 mg/L benzyladenine (BA), 0.1 mg/L gibberellic acid ( $\text{GA}_3$ ),  $10^{-3}$  mg/L 2,4-dichlorophenoxyacetic acid (2,4 D), 2% sucrose, 7–5 g/L Merck agar (art. 1613), pH 5.8.

#### 2.3.2. Shoot-Tip and Node Culture/Multiplication Medium

Quoirin and Lepoivre (QL, see Table 1) macroelements and modified microelements with  $10^{-1}$  mM Fe ( $\text{FeNa}_2$  EDTA), Walkey’s vitamins (9), 100 mg/L l-methionine and l-tyrosine amino acids, 1 mg/L BA, 0.1 mg/L indolebutyric acid ( $\text{IBA}_{\text{KOH}}$ ), a carbohydrate solution resulting from a previous autoclaving of the sucrose (3%) in the presence of activated charcoal (1%) (10), pH 5.5, 5 g/L Pastagar B (Bio-Rad, Belgium).

#### 2.3.3. Elongation Medium

Half-strength QL macroelements, full strength modified microelements with  $10^{-1}$  mM Fe ( $\text{FeNa}_2$  EDTA), Walkey’s vitamins,  $\text{GA}_3$  (1 mg/L), sucrose (2%), pH 5.5, 5 g/L Pastagar B (Bio-Rad, Belgium).

**Table 1**  
**Formulations of the culture media components**

<i>Macroelements (g/L)</i>		
	Murashige Skoog (6)	Quoirin Lepoivre
NH <sub>4</sub> NO <sub>3</sub>	1.65	0.4
KNO <sub>3</sub>	1.9	1.8
CaC <sub>12</sub> × 2H <sub>2</sub> O	0.44	–
MgSO <sub>4</sub> × 7H <sub>2</sub> O	0.37	0.36
KH <sub>2</sub> PO <sub>4</sub>	0.17	0.27
Ca(NO <sub>3</sub> ) <sub>2</sub> × 4H <sub>2</sub> O	–	1.2
<i>Microelements (mg/L)</i>		
	Nitsch (7)	Modified Quoirin Lepoivre
ZnSO <sub>4</sub> × 7H <sub>2</sub> O	10	8.6
H <sub>3</sub> BO <sub>3</sub>	10	12
MnSO <sub>4</sub> × 4H <sub>2</sub> O	25	1
CuSO <sub>4</sub> × 5H <sub>2</sub> O	0.025	0.025
KI	–	0.08
Na <sub>2</sub> MoO <sub>4</sub> × 2H <sub>2</sub> O	0.25	0.25
CoC <sub>12</sub> × 6H <sub>2</sub> O	–	0.025
<i>Vitamins (mg/L)</i>		
	Jacquot (8)	Walkey (9)
Thiamine	1	0.4
Ca Panthotenate	0.5	–
Biotine	0.1	–
Meso-inositol	500	100
Riboflavine	0.1	–
p-Aminobenzoic acid	1	–
Nicotinic acid	1	–
Folic acid	0.01	–

#### 2.3.4. Rooting Medium

“NK” rooting medium is our reference medium to root woody *Rosaceae* species (11–13). It consists of a basal rooting medium (BR): 1/2 QL macronutrients and full strength modified microelements with  $2 \times 10^{-1}$  mM Fe (FeNa<sub>2</sub>EDTA), Walkey’s vitamins, 2 mg/L IBA<sub>KOH</sub>, 2% sucrose, pH adjusted to 5.6, 5 g/L Pastagar B (Bio-Rad, Belgium), completed (according to genotype requirements) by a “NK” complex of vitamins and amino acid containing 1 mg/L riboflavin, 0.1 mg/L pyridoxine, 25 mg/L nicotinic acid, 1 mg/L Ca panthothenate, 1 mg/L ascorbic acid, and 5 mL l<sup>-1</sup> water-soluble vitamin D<sub>2</sub>, 5 mg/L vitamin K<sub>5</sub> and 100 mg/L L-proline. Stock auxin solution is prepared by dissolving IBA (Sigma) with KOH 1 N.

#### 2.4. Acclimation to Ex Vitro Conditions

1. Greenhouse facilities: benches covered with plastic foils, sun protection, watering and spray equipment, temperature regulation systems.
2. Potting substrate consisting of compost and grade 3 vermiculite (Sibli, Belgium) (2:1 by volume).

### 3. Methods

#### 3.1. Preparation of the Culture Media

1. Prepare the culture media according to the formulations properly given in Tables 1 and 2 and descriptions in the text above by diluting and mixing the macronutrients and micronutrients issued from 10 and 1,000 times concentrated stock solutions.
2. Adjust the pH to 5.5 with KOH and/or HCl 1 N solutions.
3. Add 0.5% (W:V) Pastagar B (except Merck I613 for meristem-tip culture and other technical agar brands for large-scale production).
4. Add 2% (W:V) sucrose (except 3% sucrose solution for multiplication medium).
5. Melt agar at 90°C on heating magnetic stirrer till the medium becomes translucent.
6. Display the nutrient media in corresponding volume into suitable containers according to the growth steps: 2.5 mL in “Kimax” tubes, 15 mL in “Sigma” tubes, 165 in 500 mL-glass jars.
7. Sterilize the media by autoclaving at 110°C for 30 min.
8. Store the sterile culture media in complete darkness at room temperature when to be used shortly after preparation (max. 2 weeks) or in cold room (+2°C) for a few months of storage.

#### 3.2. In Vitro Establishment

*Prunus* sp. are established in vitro from meristem-tips (Fig. 1a), shoot tips or nodal buds, the initial explant size ranging from 0.1 mm to a few cm respectively (see Note 2).

##### 3.2.1. Meristem-Tip Culture from Dormant Buds

Explant Source

Disinfection

1. Collect annual dormant twigs from common orchard trees (see Note 3).
2. Cut the twigs into three node-fragments (TF), powder with a large spectrum fungicide (Captan or thirame (TMTD)) and store them in plastic bags at +2°C for maximum 5 months.
3. Immerse the TF in ethanol (94°) and shake for 5 min with magnetic stirrer.
4. Transfer and shake the TF into Ca hypochlorite (9%) solution (dilution with reverse-osmosis water on a magnetic stirrer for 15 min and filtration through a Whatman N°1 filter paper placed in a 20 cm Ø funnel. The filtrate can be used during 1 week when stored at room temperature) for 20 min and shake well during the treatments.
5. Transfer the TF in a sterile beaker with flamed tweezers, rinse three times in sterile distilled water (reverse-osmosis water is autoclaved at 120°C for 30 min and kept sterile in 0.25–0.5 L screw capped bottles. 200 mL sterile water is necessary/cultivar) and keep the TF in sterile water until meristem-tip excision.

## Excision (see Note 4)

6. In a quiet corner of a room where laminar flow equipment is continuously working or on the bench of the laminar flow cabinet gather together a stereomicroscope, 1 scalpel, 2 small size forceps, and 2 watch chucks mounted with pieces of razor blade.
7. Tools are dipped into alcohol (70%), flamed and kept sterile between two pieces of “Joseph paper” previously autoclaved (see Note 5).
8. Remove with sterile scalpel the bark around the vegetative bud.
9. Under stereomicroscope (magnification rate:  $\times 12$ ), progress into the vegetative bud by removing scales sequentially with the first forceps and leaflets afterwards with the second one until the meristematic dome with leaves primordia appear.
10. Cut off with razor blade fragment the last leaflets and primordia that prevent the meristematic dome from easy access.
11. Excise with the second razor blade the meristematic dome (0.1 mm long) for virus elimination or the meristematic dome with one or two leaf primordia.
12. Transfer quickly the explant (to prevent it from desiccation) into appropriate gelled medium kept layered in “Kimax<sup>®</sup>” tubes (two explants/tube).
13. Flame the tube aperture and screw it enough to avoid contamination while enabling gas exchange.
14. Expose the meristem-tips to Sylvania gro lux light (see Note 6).

### 3.2.2. Shoot Tip and Node Culture

## Explant source

1. Collect actively growing shoots ( $\geq 3$ –5 cm long) from axis or laterals of forced twigs (see Note 7); of mother plants growing under dry conditions in glasshouse or in the field (shortly after flushing) (see Note 8).
2. Remove the leaves blades and prevent the shoots from wilting by storing them inside clean plastic bags.

## Disinfection

3. Once in the laboratory, prepare a 500 mL container with screw cap, containing a glass support and a filter paper (see Fig. 1b).
4. Totally remove the putative resting leaf blades while keeping the petioles, cut the stem into pieces of three nodes length and put them lying onto the glass support.
5. Let 1 mL formaldehyde solution (37%) soaking the filter paper and screw the jar for 15 min.
6. After such exposure to formaldehyde vapors, open and move the filter paper to ammoniac solution.

## Excision

7. Limit the explant size to the middle node(s) of the shoot fragments, keeping the stem situated under the bud as long as

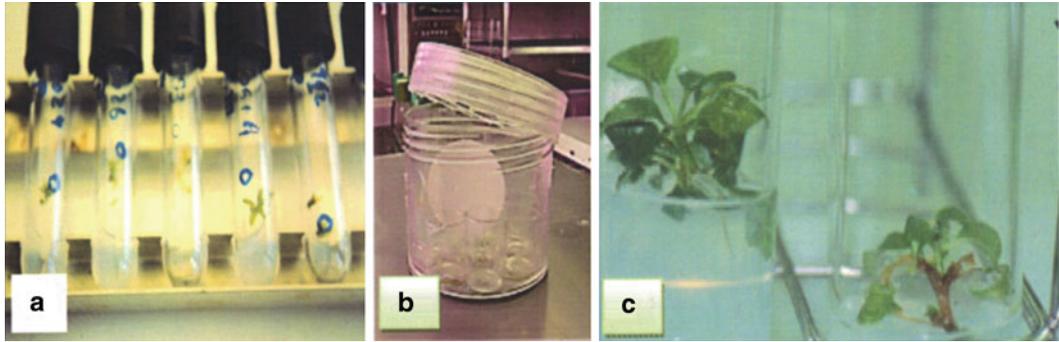


Fig. 1. Meristem-tip forming rosettes (a), tools ready for shoot-tip disinfection by formaldehyde vapors (b) and detection of *Prunus* "necrotic ring spot" virus infected mericlone after micro-grafting and necrosis of "Shirofugen" indicator (c).

possible while the upper part as short as possible respectively, and discard the distal part of the leaf petiole.

8. Transfer the nodes and apices ( $\pm 1$  cm long) to "Sigma" culture tubes containing multiplication medium.
9. Cultivate these explants under Sylvania gro lux lighting (see Note 9).

### 3.2.3. Excision of Meristem-Tips from In Vitro-Shoot Apices

1. Cut off the elongated shoots one by one from clusters and progress into the growing apex using successively forceps and razor blade fragment mounted on a watch chuck until the meristematic dome appears.
2. Proceed like described from the step 11 for initial meristem-tip establishment (see Subheading 3.2.1).

### 3.3. Multiplication by axillary branching

Axillary branching is induced from pre-formed buds of leafy rosettes (see Note 10), shoot apices ( $\pm 1$  cm size), fragments of leafy shoots, and fragmented clusters.

1. Check the sanitary state of the culture, looking for fungal/yeast contaminations at the surface of the culture medium and/or by transparency for bacteria at the interface between plant material and culture medium (see Note 11).
2. Pick up the clusters one by one to single Petri dish or sterile paper sheet using one tool set per cluster.
3. Totally remove calluses or dubious cell proliferations from any origin (see Note 12).
4. Individualize rosettes and shoots.
5. Cut off the shoot apices from leafy fragments and protect them from air desiccation.
6. Insert both rosettes and apices ( $\pm 1$  cm long) upright with half upper part outside the gelled medium.

7. In other single jar, layer the leafy shoot fragments at the surface of the fresh medium and insert cluster fragments upright.
8. Expose the culture to daylight or gro lux lighting for 1 month (see Note 13).
9. Follow up the plant material for growth (see Note 14; Fig. 2a); multiplication behavior (see Note 15) and putative occurrence of physiological disorders (hyperhydricity and apex necrosis) (see Note 16).

### 3.4. Shoot Elongation

Shoot elongation is a pre-requisite culture step for high rooting performances and for optimal plantlet quality (see Note 17; Fig. 2).

1. Check by transparency the sanitary state of the culture, looking for fungal/yeast and/or for bacterial contaminations (see Note 11).
2. Set up an elongation method according to the genotype susceptibility to hyperhydricity:
  - *For susceptible genotypes*: clusters are transplanted to a gelled elongation medium.
  - *For low susceptible genotypes*: a liquid elongation medium is added onto the multiplication medium (as a double phase), covering half of the shoots height of the clusters (see Note 18).
3. Apply the elongation treatment from the third week of the last multiplication cycle (according to the genotype) under gro lux lighting.
4. Follow up the behavior of the plant material (see Fig. 2b) in a way to anticipate apex necrosis as the main physiological disorder that also affects the shoots later on (until roots elongation) (see Note 16).

### 3.5. Rooting

Rooting efficiency depends on the previous culture conditions and shoot handling applied (see Note 17).

1. Check by transparency the sanitary state of the culture, looking for fungal, yeast and/or for bacterial contaminations (see Note 11).
2. Set up the appropriate rooting design according to genotypes requirements about medium composition and environmental culture conditions (see Table 2; Note 19).
3. Collect 2 cm-long (at least) shoots vigorously growing after tufts elongation. Cut them with a scalpel at their base lowest part and remove the leaves that could prevent the shoot insertion into the gelled medium (see Note 20) without damaging the stem tissues. Turn the cluster back into the elongation medium for growing additional shoots (see Note 18).

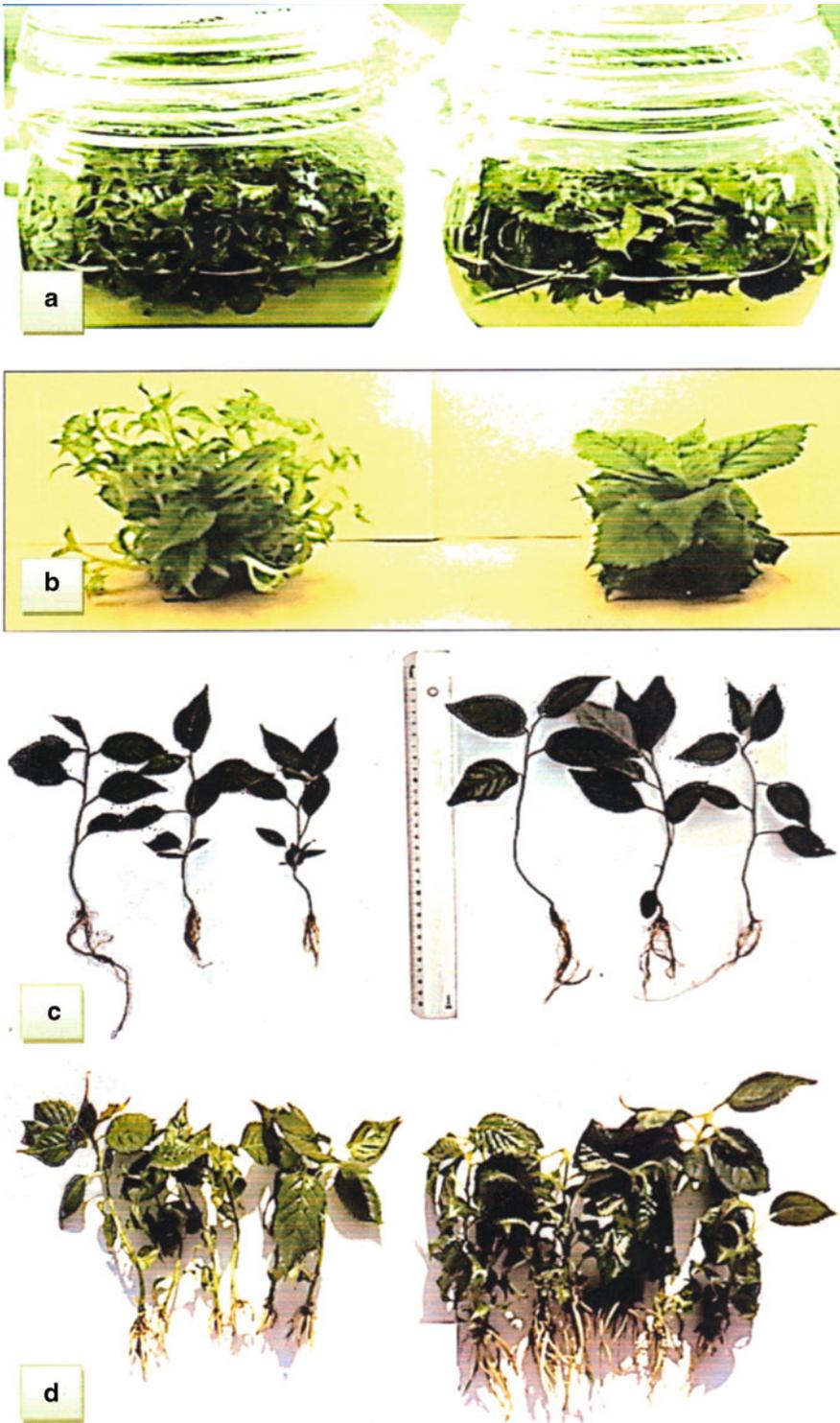


Fig. 2. Interferences between propagation conditions and final plant quality: example of *P. accolade*. Comparison of the basal multiplication medium (*left*) with the same medium without IBA 0.1 mg/l-1, methionine and tyrosine (*right*). On the left: higher number of axillary buds (a) and elongated shoots (b) after a "double layer" procedure, but formation of similar number and less vigorous roots (c) and less vigorous growth in acclimation (d).

**Table 2**  
**Rooting conditions for the best performances of different cherry cultivars and related *Prunus* species**

Variety	Mericlone	Medium tested <sup>a</sup>	Darkness (days)	Total plants	Rooting rate (%)	
					Average	Maximum
<i>P. avium</i>						
cv. Bing	1	PN 100 (3)	7	472	43.0	54.0
cv. Burlat	1	BR (3)	–	399	49.3	76.2
cv. Helshoven	1	NK (2)	–	255	48.2	52.4
cv. Lambert spur	1	BR	–	100	54.0	54.0
cv. Napoleon	1	NK (2)	–	195	46.7	77.1
cv. Sam	1	BR	12	250	39.8	74.0
cv. Schneider	2	BR (5)	12	599	56.5	83.8
cv. Van	1	BR	8	497	57.1	60.3
<i>P. cerasus</i>						
cv. Montmorency	8	NK (4)	5	1394	41.2	95.7
“Austera”	4	BR	–	273	89.9	100
“Rhexii”	23	BR	–	1444	19.8	57.1
Cherry rootstocks						
<i>P. avium</i> F12/1	2	PN 100 (2)	–	102	81.2	93.3
<i>P. dawycensis</i> “Damil”	13	BR	3	2930	90.1	98.5
<i>P. canescens</i> “Camil”	2	BR (4)	–	1855	77.0	95.2
<i>P. incisa</i> × <i>serrula</i> “Inmil”	2	BR	–	3270	97.2	100
<i>P. cerasifera</i> “Myrobolan”	4	BR	0 5	1063	40.8	80.3
		Ind. BR liquid	–	398	52.0	82.8
<i>Ornamental Prunus</i> sp.						
<i>Prunus accolade</i>	10	BR (1)	–	232	87.1	100
<i>P. Hally jolivette</i>	1	BR	–	2143	89.6	98.2
<i>Prunus fruticosa</i>	2	BR	–	189	86.1	90.3
<i>Prunus kurilensis</i>	35	BR	–	2069	80.1	100
<i>Prunus kursar</i>	13	BR (3)	–	2001	74.8	100
<i>Prunus pandora</i>	2	BR	–	103	88.6	93.1
<i>P. piliosuscula</i>	4	BR	–	616	47.3	100
<i>P. serrulata</i> Lindl. “Daikoku”	6	BR	–	769	98.0	100
“Gioïko”	4	BR	5	267	69.0	98.0
“Judii”	28	BR (7)	–	2156	98.3	100
“Kanzan”	2	BR	4	1529	87.5	99.0
“Kokonoye”	30	BR (3)	–	1283	94.1	100
“Okiku”	7	BR (2)	–	524	75.0	100
<i>P. yedoensis</i> “Moerheimii”	4	BR	–	552	94.2	100
“Shidare yoshino”	3	BR	–	292	88.6	100
<i>Wild cherry clones</i>						
<i>P. avium</i> clone B20	1	IPN50	–	343	82.2	89.4
BB1	1	NK	5	190	96.8	96.8
G1	1	BR	8	195	98.0	98.0
G26	1	PN100	9	126	75.4	75.4

(continued)

**Table 2**  
**(continued)**

Variety	Mericlone	Medium tested <sup>a</sup>	Darkness (days)	Total plants	Rooting rate (%)	
					Average	Maximum
M15	1	PN100	11	239	90.4	90.4
MB1	1	IPN50	10	53	54.7	54.7
S2	2	BR	–	183	59.5	91.6
S4	1	PN100	–	563	83.4	84.2
SA2	1	BR	–	210	32.4	33.0

<sup>a</sup>Number of rooting conditions tested

BR: basal rooting medium; NK: Basal rooting medium+ NK complex; PN100: “NK” medium with Nicotinic acid (100 mg/L); PN50: PN100 medium with nicotinic acid (50 mg/L), IBA (1 mg/L) and NAA (0.1 mg/L)

4. Prevent desiccation by keeping the shoots in closed Petri dish while collecting all the others.
5. Insert  $\pm 0.8$  cm of the stem base into the rooting medium.
6. Darkness treatment corresponding to the root induction stage (14) is carried out at room temperature and photoperiod; culture corresponding to the root elongation stage takes suitably place under Sylvania gro lux lighting.
7. Follow up the plant material during 20–30 days, checking root formation and apex necrosis (see Note 21).

### **3.6. Acclimation and Transfer to the Field**

1. Collect the rooted plantlets when the roots reach  $\pm 2$  cm long (see Note 22).
2. Remove gel from the roots with tap water.
3. Size and accommodate the plantlets into plastic boxes, water slightly the leaves before closing.
4. Store at  $+4^{\circ}\text{C}$  for 10 days before ex vitro acclimation (see Note 23).
5. Transfer to well-drained substrate (compost and vermiculite, 2:1 vol) previously powdered and mixed with large spectrum fungicide (Captan) (see Note 24), keep high temperature ( $23^{\circ}\text{C}$ ) and long days to re-establish the photosynthesis activity.
6. Spray with a large spectrum fungicide (Captan, TMTD) at low concentration ( $5\text{ g l}^{-1}$ ).
7. Cover with polyethylene sheet during the operations of soil transplantation.
8. Avoid direct sun exposure of the plantlets and prevent atmosphere overheating inside, keep it completely closed (100%

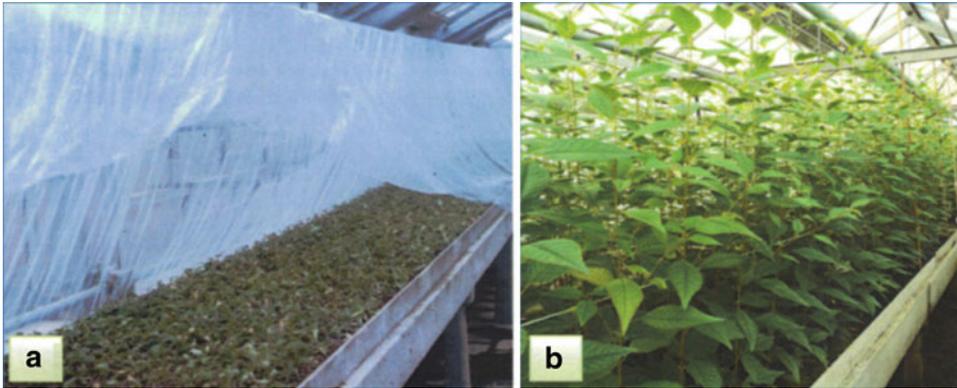


Fig. 3. “Damil” dwarfing cherry rootstock in acclimation conditions (a) and growth after 3 months in greenhouse (b) under water-saturated atmosphere conditions.



Fig. 4. Flowering of own-rooted Montmorency sour cherry cultivar, 5 years after in vitro culture.

relative humidity in the atmosphere) until apices re-growth is attested (see Fig. 3).

9. Remove the polyethylene sheet progressively and water the leaves during adaptation to the greenhouse atmosphere.
10. Transfer 3 month-old well growing plantlets to cold room (+4°C), in the dark for 3 weeks before direct culture in the nursery (see Note 25).

Rootstock plants are trained for grafting next year (see Note 26) or edged for healthy cuttings production (see Note 27). Own-rooted cultivars are healthy scion sources but are also used for sour cherry production (*P. cerasus* sp.) (see Fig. 4).

## 4. Notes

1. In addition to the maintenance of the laboratory's equipment, it is of extreme importance to practice in the laboratory strict rules of operations. Bacteria and fungi are transported from the environment during explant handling. Therefore, clean regularly:
  - The benches of the laminar flow cabinets (before, during, and after the plant manipulations) with 70% alcohol solution.
  - The external surface of the plant containers before bringing them into laminar flow.
  - The hands of the operator and flame heavily the tools (2 sets of scalpels and forceps always available) at every shoot cluster handling.

Clean the shelves of the growth chamber too and prevent from contamination due to dust accumulation and the presence of mites. Take into account temperature gradients naturally existing between shelves when displaying the cultures through the culture room and avoid overheating which causes condensation inside the jars.

2. Meristem-tip explant is required in case of cleaning the plant material from viruses (4). It is also the most suitable explant when cultures are initiated from field-growing mother trees (putatively contaminated). “Prunus Necrotic Ring Spot Virus (NRSV)”-contaminated material also should be easily screened during *in vitro* culture a few days after micro-grafting on “Shirofugen” indicator (see Fig. 1c). Leafy primordia reduce the virus elimination. *In vitro* thermotherapy treatment of shoots, combined to meristem-tip culture is required for the eradication of NRSV and “Prune Dwarf” viruses. Shoot tips and nodes from growing shoots should be considered only when the mother tree is certified free of any pathogens. Pre-grafting the healthy material onto greenhouse's growing rootstocks provides longer-term supply of explants.
3. *Where from?* Collect from the tree crown avoiding contact with the soil. The contamination rate is kept under 5% when the twigs directly originated from the mother-tree crown, but it rises to 90% when issued from stool beds and after pruning. *When?* From the end of autumn to the post-dormancy stage. *Transport to the laboratory?* Use clean plastic bags and prevent from overheating.
 

Explant survival increases with bud dormancy decline and explant size increase. Cold storage (1 month at +2°C) breaks the bud dormancy and extends the establishment period. That is our basic protocol for all *Rosaceae* species (11, 12, 15).

Once established, healthy plant material should be advantageously stored at +2°C as germplasm line for quick start of a new plant production till 3 years later (16).

4. Meristem excision requires qualified people for optimal management and adequate sizing of the explants along the day. A skilled technician is able to excise 60 meristem-tips a day.
5. “Joseph paper” is a light-weight paper towel (25 g/m<sup>2</sup>) originally used in the eighteenth century to dry and pack valuables and used for very pure filtering, as well. Actually this paper is commonly used in laboratories for cleaning and drying glass tubes, microscope slides, bottles, etc.
6. Primordia, bud, and rosette developments are successive stages to follow at the stereomicroscope. The formation of a complete leafy “rosette” (see Fig. 1a) requires 1–3 monthly culture according to the genotype. Transferring the rosette without tissue injuries prevents the plant material from putative hyperhydricity problems.
7. Prefer annual twigs at the post-dormancy stage for forcing.  
*Forcing conditions?* Prepare a glass jar containing a water solution of 200 mg/L 8-hydroxyquinoline sulphate, pH adjusted to 5.6. Put the twigs at room temperature, with their basal part immersed in the solution that is refreshed every week.
8. Choose the shoots less exposed to contamination (inside the tree crown for example). Avoid contact with any soil particle. The surface of the plant material has to be dry for preventing the detrimental effect of formaldehyde vapors mixing with water during the disinfection treatment.
9. Apexes and buds grow into foliated rosettes and shoots within 1–3 months according to the growth season and relevant physiological correlations between buds. Monthly subcultures are performed tube by tube with extreme care of transmitting contaminations from latent bacteria, fungi.
10. “Rosette” (=growing apex with expanded leaves) is the minimum explant size to consider for transplantation. Excess of tissue injuries induces hyperhydricity.
11. *Visual detection of bacterial contamination?* Generally a veil appears at the interface between the plant tissue and the culture medium, but the bacteria could spread on the surface all over the culture medium. This could further cause the complete inhibition of root formation and significant mortality of the plantlets during acclimation.  
*How to recover healthy lines from bacterial contaminated material?* Clusters kept in their original container and placed in the dark during multiplication or elongation stages grow into etiolated shoots within ±12 days. Their shoot tips

( $\leq 0.5$  cm) are then excised and isolated on a bacteria detection medium. Those who are reported healthy after 15 days are transplanted for new multiplication cycles.

12. Friable and/or compact calluses form at the twig's cut end inserted in the culture medium. Remove them before transplantation to exclude putative adventitious regeneration and to insure a genetic conformity of the plant material exclusively from axillary origin.
13. Recalcitrant genotypes should be managed in 6 week-multiplication cycles with a first transfer of the clusters to a fresh medium after 3 weeks of culture.
14. Two distinct growth phases are observed during multiplication:
  - “Budburst”: from the beginning of the culture, the axillary buds start to develop leafy rosettes due to cytokinin effect.
  - “Shoot development”: from the third week of culture, the internodes start to elongate and form shoots when cytokinin budding effect declines.
  - Most of the main shoot apices temporarily cease growing whereas axillary buds start bursting.
15. On average, the variability of the monthly rate of multiplication may exceed 40% from a subculture to another one. Very strong at first, it gradually fades with repeated cultures in the presence of BA (simulating a kind of habituation effect to cytokinin) and a clear reduction in the number of shoots produced is observed. In *P. avium*, such variability is supposed to have a genetic cause. That is even persisting after several tens of subcultures performed in strictly uniform culture conditions.

*How to manage?* Regular changes in the composition of the culture medium (increase or decrease of macronutrient concentration, higher Ca nitrate, and K phosphate that improve multiplication while Mg sulphate promotes shoot elongation, addition of vitamin D2 or some amino acids, etc.) usually should increase significantly the rate within a three subculture period of time.

Particular attention has to be paid to regulate the subculture cycle according to bud bursting and elongation. Short interruption by storing the clusters for a minimum of 3 weeks at  $+4^{\circ}\text{C}$  (until 6 months in continuous darkness) at the end of any subculture favors normal axillary branching performances.

Such stimulation means are important when planning commercial plant production.

16. “Hyperhydricity” and “apex necrosis” are both physiological disorders that disturb the whole micropropagation process. *Hyperhydricity*: appears after the shoot endogenous regulation loses the control of BA growth effect. If that already occurs

sometimes during the establishment step, it is mainly expressed during multiplication stage with increasing the number of subcultures and affects shoot elongation and rooting afterwards.

*Symptoms?* Curled leaves, thin limb, translucent and/or deep green, thick stem, hydric aspect of the shoot

*How to manage?* Avoid excessive shoot injuries through transfer operations. Grow the susceptible genotypes in colder sites of the culture room. Temporarily reduce the BA content of the multiplication medium that stimulates stem and leaves growth. Add 0.5–1% galactose to the multiplication medium and increase the size of explants picked from the less affected shoot clusters. Tyrosine (100 mg/L) and glutamic acid (100–200 mg/L) are helpful to maintain normal aspect of the microcuttings. Methionine (100 mg/L) improves multiplication without hyperhydricity amplification (in experiments with *P. dawnyckensis*).

*Apex necrosis:* Occurs at the end of the multiplication stage, during elongation and at the beginning of the rooting stages. Shoots regrow from axillary buds when roots elongate.

*Symptoms?* Necrosis starting from the apical leaves most often to internodes till the base of the affected shoots.

*How to manage?* Start elongation earlier, from the second week of multiplication. Regarding *P. canescens* (cherry dwarfing rootstock), necrosis rates have been reduced to less than 10% by limiting the ultimate multiplication cycle and the elongation phase to 3 and 2 weeks, respectively.

17. Shoots directly collected from the propagation medium generally produce more callus and form less and shorter adventitious roots as well. Elongation step prepare the shoots physiologically to respond to further auxin stimulus; that drastically increases rooting percentages of most species.
18. Partial immersion (see Fig. 2a) of clusters stimulates axillary bud development into shoots with long internodes. After 10–20 days of culture, 30–50% of them can be collected for rooting. Back on the elongation medium, clusters will provide second and most often third shoot yields at  $\pm 10$  day intervals. Apex necrosis and hyperhydricity will be limiting factors. The use of ethrel (2-Chloroethylphosphonic acid) (0.1%) in the liquid medium containing GA<sub>3</sub> (1 mg/L) may additionally increase the amount of buds forming shoots but it also speeds up the apex necrosis. However, liquid basal medium could be efficient enough for some cultivars. Previous culture conditions to rooting including shoot elongation method influence the quality of the plantlets (see Fig. 2).
19. Easy-to-root genotypes form roots in presence of 2 mg/L IBA<sub>KOH</sub>.

- Others require 3–5 days of darkness from the transfer, as complementary rhizogenous stimulus; the duration of the treatment being adjusted in function of the ambient temperature.
- Difficult to root genotypes require additional stimulus: vitamin D<sub>2</sub>, L-proline, riboflavin can be applied distinctly or gathered and eventually completed by other vitamins in “NK complex.”

The Table 2 reports the best rooting conditions (single adjustments of the medium composition, duration of the darkness treatment) determined for several *P. avium* varieties and wild types, *P. cerasus* and *Prunus* species used as root-stocks or ornamentals.

For a few genotypes, root induction can be achieved after 5–10 days of induction, dipping in liquid medium followed by a transfer to hormone free medium containing calcium nitrate and sucrose (at the concentrations of the BR) or to slightly watered vermiculite. Such root induced-shoots can be kept for months at low temperature (+4°C). That makes easier both acclimation and sales of the plantlets.

20. Only the first three subapical leaves (mainly the second and the third ones) are essential for rooting, although procedures like apex removal only lead to root emergence delay.
21. Adventitious roots appear between the twelfth to the twenty-fifth days of culture. Successive steps of induction, initiation, and elongation of the rooting process require different hormonal regulation obtained through the synergistic effects of physical and chemical factors. High level of auxin activity (darkness treatment and the addition of L-proline to the auxin enriched medium) is required only for induction. Too long and too strong stimulus generally leads to excess friable callus, root growth inhibition, and even interruption of shoots apical growth, which, however, can be controlled by riboflavin supplementation and light exposure (to oxidize exogenous auxin and thus reduce its action). Total absence of friable callus at the stem sectioned surface theoretically excludes inhibition as cause of rooting failure.
22. Root quality that insures good tolerance to plant handling and to soil adaptation improve plant survival. The number of roots is very important for quick restoration of an autonomous plant growth mainly for cultivars unable to form new roots ex vitro.
23. Plants could be stored at +2°C for maximum 3 weeks.
24. The use of sterilized substrate in alternative must be avoided because it can be quickly invaded by air born fungi not necessarily pathogenic but overgrowing the young plantlets.
25. When the plants fail to grow in the field, restore and synchronize the re-growth by spraying gibberellic acid (GA<sub>3</sub>: 200 mg/L).

26. Plants kept growing in closed greenhouse and water-saturated atmosphere grow faster and without growth arrest (see Fig. 4a) compared to field-growing plants. These become ready for grafting at the end of the growth season.
27. “Bonsai” training system in greenhouse is very efficient for cutting rootstocks and cultivars in healthy culture conditions. Such *in vitro* mother plants of “Camil” (*P. canescens*) and “Damil” (*P. dawnyckensis*) dwarfing rootstocks annually produce 1,000–1,500 well-rooted plants per m<sup>2</sup> for 5 years, in a low cost process repeating cutting every 4–6 weeks from April to September and mother plants re-growth with spraying gibberellic acid (GA<sub>3</sub>; 200 mg/L).

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## Micropropagation of Peach Rootstocks and Cultivars

Ildikó Balla and Lucienne Mansvelt

### Abstract

Peach (*Prunus persica* (L.) Batsch) is one of the most popular stone fruits, commercially produced largely in Mediterranean and, to a lesser extent, in continental climatic conditions. Several breeding programs with different aims release annually large numbers of new cultivars. Micropropagation offers a suitable method to provide the growers of sufficient quantities of rootstocks, as well as of pathogen-free planting material of old and new cultivars. An effective four-step micropropagation procedure for cultivar and rootstock production is described here, based on the use of modified MS and WPM media. The health status of the initial shoot tips is very important, also because the growth and proliferation rate of shoot cultures from virus-infected clones are generally very poor. Proliferation and elongation phases depend on the major macro-elements, as well as the content and ratio of plant growth regulators. It is important to grow the cultures at 22°C, as hyperhydricity may develop at higher temperatures. Although sucrose is the most common carbon source used during proliferation and rooting, for some peach cultivars and rootstocks the replacement of sucrose (10 g/L) with glucose (20 g/L) in the rooting medium improves the rooting and survival rates of plants in the acclimatization phase. The rooting rate of the rootstock “Cadaman” is improved with the chelated form of iron FeEDDHA at 150 mg/L. Rooted plants are acclimatized in greenhouse under high humidity conditions.

**Key words:** Cadaman, GF-677, Inter-specific hybrid rootstocks, PeDa, PeMa, *Prunus persica* (L.) Batsch, Shoot-tip culture, Stone fruits

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### 1. Introduction

The peach (*Prunus persica* L. Batsch), originated from China, is produced commercially in 80 countries worldwide on about 1.6 million ha. In 2010, the peach and nectarine fruit harvest was 20.3 million tons, which constitutes 53% stone fruit traded worldwide (1). Peach and nectarine cultivation is mainly restricted to the Mediterranean climates that are characterized by warm to hot, dry summers and mild, wet winters. Peach has a very long ripening period which starts from the middle of March and finishes early

November (Northern Hemisphere), and from end-September until end-March (Southern Hemisphere). Currently, there are several peach breeding programs in the USA, Europe, Latin America, Asia, and to a smaller extent in Africa and Australia, aimed to improve the characteristics of existing cultivars. Today, for instance, the plum pox virus (PPV) and the European stone fruit yellow (ESFY) phytoplasma cause the most serious losses to the peach growers; their control in infected orchards is not effective; however, possible prevention is by planting healthy peach trees. Commercial peach cultivation requires not only new cultivars, but also vegetative propagated rootstocks suited to the various production conditions.

Tissue culture techniques can provide new cultivars to meet the demand of market. The rapid multiplication of elite cultivars (2–5), production of pathogen-free plants (6–8), breeding of early ripening peach varieties (9), and gene transfer studies (10–12) are important objectives for micropropagation. The micropropagation system, independent of the growing season, is ideally suited to fulfill these requirements. The propagation of healthy material from gene collection and breeding work is fundamental especially when pathogen-free material is unavailable. Phytotherapy is a crucial step to carry out micropropagation (6–8). This will markedly improve the growth and propagation rate of clones infected by PPV and ESFY.

In case of peach, several reports are available, dealing with the development and optimization of individual steps of the micropropagation cycle, as well as of complete protocols for specific rootstocks and cultivars (2–5, 13, 14). This manuscript describes a stepwise protocol for shoot induction, propagation, elongation, rooting, and subsequent acclimatization, suitable for *in vitro* mass production to a wide range of rootstocks and cultivars with some minor modifications.

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## 2. Materials

### **2.1. Decontamination of Explants and Transfer of Tissue Culture Plants**

1. Tap water.
2. Contact fungicide solution.
3. Commercial bleach solution, diluted to have 0.7% of active chloride (see Note 1).
4. Autoclaved tap water.
5. 70% ethanol (v/v).
6. Sterile 500 mL Erlenmeyer flasks/500 mL glass bottles with wide neck (4 cm).
7. Sterilized filter paper.

8. Instruments for micropropagation: pruning shears, scalpels with blades, forceps.
9. Glass bead sterilizer.
10. Laminar flow benches.

### **2.2. Preparation of Culture Media**

1. 1 M concentrated solution of KOH and HCl for pH adjusting.
2. Chemicals of the modified MS (15), WPM (16), and Jacquot (17) formulations shown in Tables 1–4, dissolved in distilled water.
3. Glassware to measure and to mix the medium components.
4. Sterilized paper (size about 10 × 18 cm).
5. 200 mL screw cap culture vessels/magenta jars.
6. Semipermeable plastic cling wrap to close the culture vessels.
7. Double distilled water for preparing stock solutions.
8. Laboratory facilities: balances, magnetic stirrer, pH meter, microwave, autoclave, fridge.

### **2.3. Growth Room for In Vitro Culture**

1. Growth room with shelves mounted with warm white fluorescent lights, like OSRAM L 58/30 or TUNGSRAM F 29.
2. Air conditioner.

### **2.4. Acclimatization of Micropropagated Plants to Ex Vitro Conditions**

1. Greenhouse with mist benches.
2. Tap water.
3. Plastic tray “TEKU JP 3050/72” (PÖPPELMANN GmbH and Co. Pf 1160 D-49378 Lohne Germany). The tray size is 50 × 28 cm with 72 cells (4 × 3.7 × 4 cm).
4. Potting mixture consists of peat:perlite in a ratio 1:1.

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## **3. Methods**

The protocol described here follows a classic four-step micropropagation method (see Fig. 1).

### **3.1. Preparation and Sterilization of Culture Media**

1. Prepare separate stock solutions for the media according to Table 1. Store the stock solutions in a laboratory fridge at 4°C. Stock solution can be substituted with master mix powders from commercial companies by following the instruction for preparation.
2. Use stock solutions of the correct volumes to prepare three different media, i.e., establishment/elongation, shoot proliferation, and rooting medium.

To calculate the required volume of the stock solution for one liter of medium:

$$\frac{\text{required concentration (mg)}}{\text{concentration of stock solution (mg)}} \cdot 100 = x \text{ mL}$$

3. Prepare a stock solution of microelements, as indicated in Table 2. Add 1 mL/L to each medium.
4. Prepare a stock solution of Jacquot vitamins (see Table 3). Add 1 mL/L to the proliferation medium only.
5. Dissolve the correct amount of sucrose or glucose into the medium.
6. Adjust the pH according to the required medium (see Table 1), using 1 M KOH or 1 M HCl.
7. Heat the medium, add 0.6% (w:v) agar (e.g., Oxoid no. 5); continue heating and stirring the medium until the agar is dissolved.
8. Pour 50 mL warm medium into 200 mL culture vessels or 15 mL into a test tube and close.
9. Autoclave the medium at 121°C for 20 min.
10. Store the medium-containing culture vessels/test tubes in shelves. If they are kept at room temperature, discard when older than one month.

### **3.2. Stock Plants and Shoot Decontamination**

1. Collect actively growing shoot tips, about 15 cm long from 3 to 5-year-old trees, growing in pots (4-L pot is a good size) in the greenhouse (see Notes 2–5).
2. Cut back all the leaves, leaving about 0.5 cm of petioles.
3. Cut the shoots into sections, containing two buds. Discard the very weak shoot tips about 3 cm and the terminal 2-cm shoot sections. Use buds in the middle part of the shoots for tissue culture.
4. For decontamination, treat about 20 shoot sections in a 500 mL flask with 70% ethanol for 1 min, followed by immersion in the bleach solution, prepared according to section 2.1 and add few drops of wetting agent “Tween 80” to increase the efficacy. Close the flask and keep on a magnetic stirrer for 30 min (see Notes 6 and 7).
5. Transfer the flask into the laminar flow hood and rinse the shoot sections three times with autoclaved distilled water. Dry them with sterile filter paper.
6. Remove 0.2 cm of the bottom parts of the 2-bud sections using sterile instruments and put ten sections into one culture vessel.

**Table 1**  
**Culture medium composition developed for the micropropagation of “GF-677”**

Culture medium components	Establishment Elongation (mg/L)	Proliferation (mg/L)	Rooting (mg/L)	Stock solutions (mg/100 mL)
NH <sub>4</sub> NO <sub>3</sub>	1,650	200	1,650	10,000
Ca(NO <sub>3</sub> ) <sub>2</sub> ×4H <sub>2</sub> O	–	600	600	10,000
CaCl <sub>2</sub> ×2H <sub>2</sub> O	440	–	–	1,000
KNO <sub>3</sub>	1,900	900	900	10,000
MgSO <sub>4</sub> ×7 H <sub>2</sub> O	370	180	180	10,000
KH <sub>2</sub> PO <sub>4</sub>	170	140	140	10,000
FeEDDHA	–	–	120	400
FeNaEDTA	20	20	–	400
CoCl <sub>2</sub> ×6H <sub>2</sub> O	0.03	0.03	0.03	
CuSO <sub>4</sub> ×5H <sub>2</sub> O	0.03	0.03	0.03	
H <sub>3</sub> BO <sub>3</sub>	3.00	3.00	3.00	
KI	0.30	0.30	0.30	
MnSO <sub>4</sub> ×4H <sub>2</sub> O	11.00	11.00	11.00	
Na <sub>2</sub> MoO <sub>4</sub> ×2H <sub>2</sub> O	0.30	0.30	0.30	
ZnSO <sub>4</sub> ×7H <sub>2</sub> O	4.00	4.00	4.00	
Myo-inositol	100.00	500.00	100.00	1,000
Nicotinic acid	0.50	1.00	–	50
Piridoxine HCl	0.50	–	–	50
Thiamine HCl	0.10	1.00	0.40	10
Glycine	2.00	–	–	100
Ca-pantothenate		0.50	–	
Biotine		0.10	–	
Riboflavine		0.10	–	
p-Amino-benzoic-acid		1.00	–	
Folic acid		0.01	–	
IAA	1.00	–	–	10
NAA	–	0.005	–	10
IBA	–	–	0.50	10
BAP	0.06	0.50	–	10
GA <sub>3</sub>	0.10	0.25	–	10

(continued)

**Table 1**  
**(continued)**

Culture medium components	Establishment Elongation (mg/L)	Proliferation (mg/L)	Rooting (mg/L)	Stock solutions (mg/100 mL)
L-glutamine	–	50.00	50.00	1,000
Adenine sulfate	–	0.50	–	10
Glucose	30,000	–	20,000	
Sucrose	–	20,000	–	
Agar (Oxoid no. 5)	6,000	6,000	6,000	
Distilled water (mL)	1,000	1,000	1,000	
Medium pH	5.2	5.5	5.8	

The medium is based on Murashige and Skoog (15) and woody plant medium (16). Note that formulations are expressed in mg/L, while the concentrations of stock solutions are expressed in mg/100 mL

**Table 2**  
**Stock solution of microelements**

Medium components	Concentration (mg/100 mL)
ZnSO <sub>4</sub> × 7H <sub>2</sub> O	400
H <sub>3</sub> BO <sub>3</sub>	300
MnSO <sub>4</sub> × 4H <sub>2</sub> O	1,100
CuSO <sub>4</sub> × 5H <sub>2</sub> O	3
Na <sub>2</sub> MoO <sub>4</sub> × 2H <sub>2</sub> O	30
CoCl <sub>2</sub> × 6H <sub>2</sub> O	3
KI	30

**Table 3**  
**Stock solution of the Jacquot vitamins (17)**

Medium components	Concentration (mg/100 mL)
Thiamine HCl	100
Ca-pantothenate	50
Biotine	10
Riboflavine	10
p-Amino-benzoic-acid	100
Nicotinic acid	100
Folic acid	1

**Table 4**  
**Stock solution for fertilization of container grown-plants**  
**in peat-based growing medium or in rock wool**

Components for nutrition solution	Concentration (g/10 l)
$\text{NH}_4\text{NO}_3$	480
$\text{MgSO}_4 \times 7\text{H}_2\text{O}$	430
$\text{KNO}_3$	264
FeEDTA/FeEDDHA (9–10% Fe)	20
$\text{MnSO}_4 \times \text{H}_2\text{O}$	13.44
$\text{CuSO}_4 \times \text{H}_2\text{O}$	0.48
$\text{ZnSO}_4 \times 7\text{H}_2\text{O}$	0.576
$\text{Na}_2\text{B}_4\text{O}_7$	3.6
$(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \times 4\text{H}_2\text{O}$	0.07
$\text{H}_3\text{PO}_4$ (84% $\text{P}_2\text{O}_5$ )	90 mL

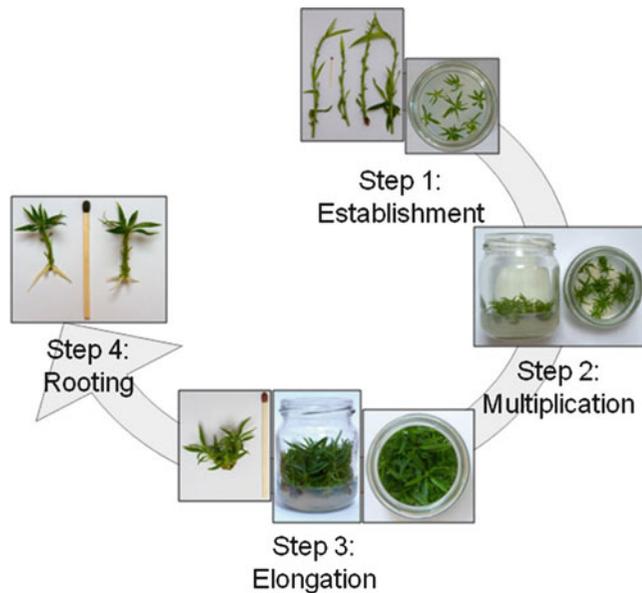


Fig. 1. The four-step method for micropropagating peach rootstocks and cultivars (Photo: P. Balla).

- Repeat the decontamination procedure the following day, except for the ethanol treatment.
- Cut 0.2 cm piece from the bottom ends of the 2-bud sections and place the 2-bud sections individually into the test tubes, then transfer them into the culture room.

### **3.3. Culture and Maintenance of Explants**

1. Use the medium prepared according to Subheading 3.1.
2. For culture establishment, place 2-bud sections into test tubes (one per tube to avoid losses due to contamination), containing the medium for culture establishment. About 100 shoot explants per genotype are necessary for successful establishment.
3. Grow the explants in a culture room at 22°C, under a 16 h light/8 h dark photoperiod, with a light intensity of 40–45  $\mu\text{mol}/\text{m}^2/\text{s}$ , provided by daylight fluorescent lamps (see Note 8).
4. Eliminate contaminated explants daily.
5. During the first 2 months, transplant cultures to fresh medium every 2 weeks.
6. Isolate shoots, developed from buds, when they are about 1 cm long, and transfer them to the propagation medium (13), 5–7 shoots per culture vessel (see Note 9).
7. Grow the cultures on the proliferation medium (see Notes 10 and 11) for about 3 weeks. Three to five new shoots start to develop during this period. Transfer the shoot clusters to the elongation medium with a fresh cut at the base, without isolating the new shoots to allow small buds and shoots to grow to about 1.5 cm long shoots which are cut from the dominant shoot. Multiplication and elongation phases alternate until the required quantity of rootable shoots are produced. On average, the culture period on the proliferation medium is 3 weeks; while on the elongation medium it is 10–14 days (see Note 12).
8. Different types of shoots develop on the elongation medium: (1) shoots shorter than 0.8 cm should be kept together and transferred to the proliferation medium for further multiplication, (2) shoots from 0.8 to 1.5 cm are suitable for further propagation, (3) the elongated shoots (about 1.5 cm) can be moved to the root induction medium (see Note 13) or back to the proliferation medium if the quantity of cultures is still not sufficient.

### **3.4. Shoot Rooting**

1. Transfer elongated shoots into vessels containing gelled root induction medium, ten shoots per vessel (see Notes 14 and 15).
2. Keep the vessels in the culture room, under the climatic conditions described in Subheading 3.3.3.
3. Generally, rooting starts in about 10 days and is completed in 21 days.

### **3.5. Acclimatization of Micropropagated Plants to Ex Vitro Conditions**

1. Remove rooted shoots with 3–5 roots (about to 0.5 cm long) from the medium. Clean root surface from the medium residuals to avoid contamination. Transfer rooted shoots into planting trays described in Subheading 2.4.3 filled with a substrate (as described in Subheading 2.4.4) for micropropagated

plantlets, wetted until run off, and place in the greenhouse (see Notes 16 and 17).

2. Spray or drench the plantlets with a fungicide (Proplant™ at the manufacturer's recommendation) in order to protect them from fungal infection.
3. Cover the plantlets with a plastic sheet to protect from direct sunshine and to keep the humidity around the plantlets close to saturation (see Note 18).
4. Shade the greenhouse in order to maintain temperature lower than 30°C, as well as to protect the plantlets from direct sunshine.
5. Water the plantlets carefully.
6. After the first 7–10 days, depending on weather conditions, open the plastic sheet gradually to accustom plantlets to the greenhouse humidity. During this time, it is important to check periodically the plants for fungal infection and, when necessary, to treat them with an appropriate fungicide, i.e., captan or benomyl.
7. After 6–8 weeks, transplant plantlets into individual containers (6×6×9 cm) filled with a mixture of sand (40%), peat (43%), alginate (15%), and organic matters (FLORASCA Hungaria) and place in open field, under irrigated and shaded conditions.
8. Fertilize the container plants 1–2 times a week with a solution (E. SZÜCS, Institute for Fruitgrowing and Ornamentals) (see Table 4 and Note 19).
9. Under continental climatic conditions, micropropagated rootstocks, acclimatized in spring, can be grafted at the end of summer/early autumn.

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#### 4. Notes

1. Commercial bleach (NaClO) is generally at a concentration of 3.5% (v/v) of active chloride.
2. Two to three weeks before isolation and establishment of explants, transfer actively growing potted plants to the greenhouse, with an interior temperature  $24 \pm 4^\circ\text{C}$ . Apply a preventative contact fungicide, e.g., captan or benomyl.
3. If potted trees are not available, cut shoots from trees in spring (April in the Northern Hemisphere, November in Southern Hemisphere) or when secondary growth occurs in July (Northern Hemisphere) under continental climatic conditions. New shoots from the middle part of the tree are suitable for establishment of tissue culture.

4. Cultivar identification and health control of the stock plants is very important especially in case of mass production (6–8).
5. Sometimes trees are maintained only in the field. In case of field-originated explants, the establishment step is time consuming, and careful and repeated disinfection treatments may be required. It is always very important to select healthy mother plants especially if field-derived.
6. In case of heavily fungal-infected explants, the bleach solution can be substituted with the more effective, but also more dangerous solution for cell viability, mercuric chloride. Use 0.5% mercuric chloride solution for 1 min. Mercuric chloride is highly toxic; hence use a dust filter mask, safety gloves, and glasses. After the use, collect this solution separately and discard under certified controlled conditions (refer to the specific instructions of your country).
7. If plants are infected with viral pathogens, such as PPV or ESFY, the disinfection treatment will not eliminate the pathogens, and phytotherapy will be required.
8. Several peach cultures are highly sensitive to the temperature of the culture room, such as “Cadaman” rootstock and “Cresthaven” and “Suncrest” cultivars. At temperatures higher than 22°C, shoot hyperhydricity can occur. Hyperhydrated cultures are swollen, the tissues are shiny and unfit for further propagation, and should be discarded.
9. The original explant often appears as a dominant shoot, surrounded by smaller shoots originated by axillary and basal buds. These de-novo formed shoots, because of their small size (from 0.2 to 0.8 cm) are still not suitable for isolation and subculturing.
10. The plant growth regulator ratio and content of the medium should be determined every time for each specific rootstock or cultivar. The medium in Table 1 was developed for micropropagation of the rootstock “GF-677,” the most widely used in peach orchards. For the multiplication of the rootstock “Cadaman,” for instance, 0.05 mg/L 1-naphthalene acetic acid (NAA) is required, while 6-benzylaminopurine (BAP) and adenine-sulfate concentrations should be reduced to 0.3 mg/L, L-glutamine to 30 mg/L, and the gibberellic acid (GA<sub>3</sub>) has to be omitted.
11. The peach cultivars propagated in our laboratory (“Babygold 6,” “Biscoe,” “Cresthaven,” “Fantasia,” “Frederica,” “Redhaven,” and “Suncrest”) require a BAP concentration of 1 mg/L and indole-3-butyric acid (IBA) at 0.05 mg/L.
12. The “PeDa,” a Hungarian *P. persica* × *P. davidiana* hybrid rootstock, requires 950 mg/L K<sub>2</sub>SO<sub>4</sub> instead of 1,900 mg/L KNO<sub>3</sub> in the elongation medium.

13. Cold storage (at 4°C) of shoots on elongation medium for 1–3 months before rooting can improve the rooting rate.
14. For the rooting medium, higher rooting percentages are achieved when FeEDTA is replaced with FeEDDHA (18).
15. The rootstock “Cadaman” requires increased potassium (1,400 mg/L) and FeEDDHA (150 mg/L), as well as reduced nitrogen (600 mg/L) concentration in the rooting medium for achieving a proper root development (2).
16. Spring time (from middle February till end of May) or September (Northern Hemisphere) is the preferable period for acclimatization under continental climatic conditions.
17. The water used for irrigation should be at greenhouse temperature.
18. The plastic sheet must not touch the plants.
19. Dilute the stock solution in Table 4 100-fold with tap water before use. For calcium nutrition, the normal tap water (used for dilution) is generally enough. Moderately hard tap water contains about 100–150 mg/L CaCO<sub>3</sub>.

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# Chapter 11

## Micropropagation of *Rubus* and *Ribes* spp.

Ewa Dzedzic and Joanna Jagła

### Abstract

Micropropagation is the most appropriate method for large-scale production of *Rubus* and *Ribes* spp. The proliferation rate of *Rubus* spp. differs in shoot tips and nodal segments. The culture media used for raspberry and blackberry propagation are MS-based supplemented with different combination and ratio of plant growth regulators, depending on the stage of culture. The initiation medium containing 0.4 mg L<sup>-1</sup> BA and 0.1 mg L<sup>-1</sup> IBA is used to stabilize shoot cultures. In multiplication media, concentration of cytokinin is doubled. In vitro rooting of shoots is achieved on media supplemented with 1.0 mg L<sup>-1</sup> IBA. *Ribes* spp. cultures are initiated from shoot tips, meristem, or dormant buds on MS medium supplemented with 2.0 mg L<sup>-1</sup> BA, 0.5 mg L<sup>-1</sup> IBA, and 0.1 mg L<sup>-1</sup> GA<sub>3</sub>. After stabilization of shoot cultures in 3–4-week time, shoot multiplication is carried out on MS medium containing 1.0 mg L<sup>-1</sup> BA and 0.1 mg L<sup>-1</sup> IBA. Shoots 2 cm long are cultured to rooting on a medium amended with 2.0 mg L<sup>-1</sup> IBA and 5.0 mg L<sup>-1</sup> IAA. Rooted plantlets are transferred to universal peat substrate and acclimatized in the greenhouse.

**Key words:** Proliferation, *Ribes*, *Rubus*, Shoot culture, Small fruits

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## 1. Introduction

Raspberry and blackberry, belonging to the genus *Rubus* (*Rosaceae*), are the most popular horticultural species cultivated in temperate climatic conditions. Traditionally, raspberry is a mid-summer crop; however, it is produced year-round with the advent of new technologies and cultivars as well as efficient and reliable transportation. Raspberry cultivars are hybrids between *Rubus idaeus* L. var. *vulgatus* (native to Europe) and *Rubus idaeus* L. var. *strigosus* (native to America). Raspberry fruits are known for high content of antioxidants (anthocyanin pigments, ellagic acid), dietary fiber, vitamins C and B; produced for both fresh market and processing. Raspberry and blackberry are commercially propagated with vegetative methods by using hard or softwood cuttings, 1-year-old suckers, layering or root cuttings. Black and purple raspberries are propagated by tip layering or tissue culture. However, several

pathogens (such as viruses, soil-borne diseases, and nematodes) hinder propagation of new selections, as traditional methods of propagation are unable to produce pathogen-free plants.

Blackcurrants (*Ribes nigrum* L.) and gooseberries (*Ribes uva-crispa* L.) are small berry-bearing shrubs, belonging to *Grossulariaceae* family, known for their edible, healthy berries, ornamental shrub habit, and flowers. The cultivation of *Ribes* fruits is aimed at both fresh and processing markets. Many *Ribes* species, especially the dark black currant types, contain high concentrations of polyphenolic compounds, notably anthocyanins and flavonols, together with high levels of ascorbic acid. Traditionally *Ribes* spp. is propagated mainly by vegetative methods: hard or softwood cuttings, layering, or green grafting dependent on weather conditions. Moreover, these techniques are unreliable to obtain healthy plant material, especially virus-free, required to establish *Ribes* spp. plantations.

Tissue culture of *Rubus* spp., established from meristem explants, eliminates viruses and enables mass production of genetically identical and healthy plant material in a relatively short time and limited space without any seasonal variation (1). The raspberry plants originated from tissue culture are used for mother stock-plant establishment or for fruit plantation, especially for early or late cropping under protected cultivation. Evaluation of micropropagated raspberry plants in the field revealed positive effect of tissue culture as propagation method on winter hardiness, fruit yield and weight, and phenotypic characteristics (2). Moreover, in vitro plants of primocane-fruiting (i.e., autumn fruiting) red raspberry are more productive than plants obtained by traditional propagation methods. However, due to the high hybridization rate between *Rubus* spp., each cultivar or even breeding clone requires the individual treatment during in vitro culture, since they respond differently to initiation, multiplication, and rooting media (3).

First information on in vitro propagation of *Rubus* dates back to the 1970s. Since then many reports documenting in vitro propagation of raspberry and blackberry cultivars have been published (1, 4–6). The in vitro response is dependent on proper explant disinfection and low propagation rates (7), and creates hindrance to the micropropagation of new cultivars (8–10). A critical step of *Rubus* micropropagation is the acclimation to ex vitro conditions and therefore the improvement of this stage of the micropropagation process is fundamental (11).

The in vitro propagation of currants and gooseberry meets today all the demands of growers, ensuring large-scale production of healthy plant material in comparatively short time. The multiplication rate of blackcurrant depends on genotype and type of explant (shoot tips or axillary buds) (12–18). Gooseberry is regarded as more difficult to micropropagate. Due to juvenile character of micropropagated plants, shoots obtained in vitro are suitable to establish stool beds to supply hardwood cutting material (19–21). This chapter describes in vitro propagation of *Rubus* and *Ribes* spp.

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## 2. Materials

### 2.1. Surface Sterilization of Source Material, Culture Media, Instrument

1. Tap water.
2. Ethanol 70% (v:v).
3. Calcium hypochlorite or sodium hypochlorite, about 35% available Cl.
4. Autoclaved reverse-osmosis water, 250 mL aliquots in 330 mL screw capped bottles.
5. 600 mL beaker (Simax glass).
6. Tissue culture facilities—instruments scalpel blades—e.g., carbon steel (sterilized by gamma radiation) Swann Morton Limited—Sheffield UK, scalpel handle, forceps, autoclaved packing paper (A5 format) or sterile plates, 70% ethanol for disinfecting hands, instrument sterilizer, laminar flow bench, magnetic stirrer, stereomicroscope, magnetic bars.
7. Stock plants either field-grown plantation or greenhouse-grown for explant source.

### 2.2. Culture Media

1. Media based on Murashige and Skoog (MS, (22)) and Lloyd and McCown (WPM, (23)) salt with modification of media formulation regarding each stage of culture: initiation, multiplication, and rooting of plant species. Media formulations are given in Table 1.
2. Culture tubes (16 mm × 100 mm).
3. Aluminum packaging foils (thickness—0.04 mm).
4. Glass beaker (500 mL).
5. Glass jars with autoclavable lids (200 or 330 mL volume).
6. Microwave oven.

### 2.3. Acclimation of Regenerated Plants to Ex Vitro Conditions

1. Tap water.
2. Plastic multiplates (2 × 2 cm, holes × 56), pots. Potting substrate consisting of peat and sand or perlite 3:1 (v:v) and fertilizer PG mix.
3. Transparent polyethylene cover.

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## 3. Methods

### 3.1. Raspberries and Blackberries (*Rubus* spp.)

Establishment and maintenance of *Rubus* spp. culture depends on many factors. For the culture initiation from virus-free plants, meristems (0.3–0.5 mm) are isolated, while from infected plants apical meristem excision must be reduced to 0.2 mm. There is a

**Table 1**  
**Culture media for *Rubus* spp. and *Ribes* spp. at initiation, multiplication, and rooting stages based on the formulation of Murashige and Skoog (MS, (22)) and Lloyd and McCown (WPM, (23))**

Component	<i>Ribes</i> spp.		<i>Rubus</i> spp.		
	Initiation and multiplication stages (MS)	Rooting stage (WPM)	Initiation stage (MS)	Multiplication stage (MS)	Rooting stage (MS)
	Concentration (mg L <sup>-1</sup> )				
CaCl <sub>2</sub>	332.02	72.5	332.02	332.02	332.02
NH <sub>4</sub> NO <sub>3</sub>	1650.0	400.0	412.5	825.0	825.0
Ca(NO <sub>3</sub> ) <sub>2</sub> ·2H <sub>2</sub> O	–	471.26	–	–	–
KNO <sub>3</sub>	1900.0	–	475.0	950.0	950.0
K <sub>2</sub> SO <sub>4</sub>	–	990.00	–	–	–
CoCl <sub>2</sub> ·6H <sub>2</sub> O	0.025	–	0.025	0.025	0.025
KH <sub>2</sub> PO <sub>4</sub>	170.0	170.0	170.0	170.0	170.0
H <sub>3</sub> BO <sub>3</sub>	6.2	6.2	6.2	6.2	6.2
KJ	0.83	–	0.83	0.83	0.83
Na <sub>2</sub> MoO <sub>4</sub> ·2H <sub>2</sub> O	0.25	0.25	0.25	0.25	0.25
MgSO <sub>4</sub> ·4H <sub>2</sub> O	180.54	180.54	180.54	361.0	180.54
MnSO <sub>4</sub> ·H <sub>2</sub> O	16.90	22.30	16.90	16.90	16.90
CuSO <sub>4</sub> ·7H <sub>2</sub> O	0.025	0.25	0.025	0.025	0.025
ZnSO <sub>4</sub> ·7H <sub>2</sub> O	8.6	8.6	8.6	8.6	8.6
FeNaEDTA	73.4	73.4	36.7	36.7	73.4
Glycine	2.00	2.0	2.0	2.0	2.0
Myo-inositol	100.0	100.0	100.0	100.0	100.0
Ascorbic acid	–	–	50.0	50.0	50.0
Nicotinic acid	0.5	0.5	0.5	0.5	0.5
Pyridoxine HCl	0.5	0.5	0.5	0.5	0.5
Thiamine HCl	0.5	1.0	0.5	0.5	0.5
Add for initiation media	BA 2.0, IBA 0.5				
GA <sub>3</sub> 0.1	BA 0.4, IBA 0.1				
Add for multiplication media	BA 1.0, IBA 0.1		BA 0.8–1.0		
IBA 0.1					
Add for rooting media	IBA 2.0, IAA 5.0				IBA 1.0

*Note:* For all media, add: sucrose 30,000 mg L<sup>-1</sup>, agar 6,000 mg L<sup>-1</sup>, adjust pH to 5.7

difference in proliferation rate in shoot tips and nodal segments. Directly after establishing the cultures, shoots proliferate very poorly, but coefficient of shoot multiplication increases with the successive subculture. The inorganic components in MS salt medium are too rich for raspberry plants. Therefore, the ammonium and potassium ions are reduced to  $\frac{1}{4}$  for initiation and to  $\frac{1}{2}$  for multiplication and rooting (Table 1). The rooting of shoots *ex vitro* in sand and peat mixture is very well suitable, more practical, cost-effective, and provides better quality of roots—more branched, stronger, and longer.

### **3.2. Preparation and Sterilization of Culture Media**

1. Prepare media according to the formulation using commercial salts or previously prepared stock solutions (macro- and microelements). Take into account the final concentration of components. Composition and concentration of all ingredients are given in Table 1.
2. Add other components, such as sucrose, vitamins, and plant growth regulators, to the required final concentration.
3. Adjust the pH of the media to 5.7 using 1 M HCl or 1 M KOH.
4. Add 0.6% (w:v) agar.
5. Dispense 20 mL initiation medium in 16 mm × 100 mm culture tubes and close with aluminum foil.
6. Dispense the multiplication and rooting media into jars, pouring 25 mL medium into each vessel. Put the lids on.
7. Sterilize media directly after preparation by autoclaving at 121°C for 20–25 min (118 Kpa steam pressure).
8. Store the autoclaved media at the room temperature in the darkness for a maximum of 1 month.

### **3.3. Plant Source Material and Surface Sterilization**

1. Take 1-year-old shoots from virus-free mother stock plants, maintained in tunnel. Divide shoots into 2 cm sections, each with a single axillary bud.
2. Rinse explants with running tap water for 30–45 min (see Note 1) (Fig. 1a).
3. Sterilize explants with 10–12% (v:v) calcium hypochlorite solution, add a few drops of commercial washing liquid, for 10–12 min in sterile jar on a magnetic stirrer (see Note 2) (Fig. 1b).
4. Rinse explants three times with sterile reverse-osmosis water and keep submerged in water prior to trimming and culture.

### **3.4. Culture and Maintenance of Explants**

1. Excise meristems from the axillary buds using a stereomicroscope under the laminar flow hood, and place the meristems onto the initiation medium (see Note 3).
2. Place meristems separately in culture tubes, containing 10–15 mL initiation medium. Prepare minimum 50 explants per genotype.



Fig. 1. Raspberry shoot regeneration in tissue culture. (a) Raspberry axillary bud on 1-year shoot, used as an explant for establishing the cultures, (b) sterilization of plant segments with 12% calcium hypochlorite, (c) 3-week-old microplant on MS medium with addition of  $0.4 \text{ mg L}^{-1}$  BA and  $0.1 \text{ mg L}^{-1}$  IBA (bar = 1.6 cm), (d) multiplied shoots on MS-based medium with addition of  $0.8 \text{ mg L}^{-1}$  BA and  $0.1 \text{ mg L}^{-1}$  IBA (bar = 1 cm), (e) induction of roots on MS medium containing  $1.0 \text{ mg L}^{-1}$  IBA, 2 weeks after being transferred on the medium (bar = 1 cm), (f) rooted “Polka” raspberry plantlet (bar = 1.5 cm), (g) raspberry plants after acclimation (bar = 5.0 cm), (h) single raspberry plant, taken out from multiplates (bar = 4.0 cm), (i) raspberry plants already potted (bar = 3.5 cm).

3. Cover tubes with sterile foil and incubate cultures for 3–4 weeks in the growth room at  $24/22^\circ\text{C}$  with a 16 h photoperiod and light intensity of about  $70 \mu\text{mol m}^{-2} \text{ s}^{-1}$ , white fluorescent light; 18 W/33 Philips TLD, Philips Electronics (see Note 4).
4. Discard contaminated explant cultures and transfer healthy, vital looking explants onto the fresh medium of the same composition (Fig. 1c).

### 3.5. Multiplication of Shoots

1. Transfer stabilized explants on the multiplication MS medium, containing 0.8–1.0 mg L<sup>-1</sup> 6-benzyladenine (BA) and 0.1 mg L<sup>-1</sup> indole-3-butyric acid (IBA) (7–8 shoots per one jar) (see Note 5) (Fig. 1d).
2. Subculture and subdivide the rosettes at 4-week intervals to fresh medium, eliminating the weak rosettes. Transfer shoots shorter than 2 cm to multiplication medium for shoot multiplication (see Note 6).
3. When the regenerated shoots show sign of chlorosis, transfer them onto the medium with doubled content of iron (see Note 7).

### 3.6. In Vitro Rooting of Regenerated Shoots

1. Transfer shoots longer than 2 cm to the rooting medium containing 1.0 mg L<sup>-1</sup> IBA (for recalcitrant genotypes 2.0 mg L<sup>-1</sup> IBA is recommended), placing ten shoots in each 330-mL jar (see Note 8) (Fig. 1e).
2. Incubate cultures at 24/22°C, 16 h photoperiod, light intensity of 70 μmol m<sup>-2</sup> s<sup>-1</sup>, white fluorescent light; 18 W/33 Philips TLD, Philips Electronics.

### 3.7. Acclimation of Regenerated Shoots to Ex Vitro Conditions

1. Remove the rooted shoots from the jars and wash roots carefully with water (Fig. 1f).
2. Transfer plantlets individually into multiwell plates, 5–6 cm, containing mixture of peat-sand or peat-perlite 3:1 (v:v) (Fig. 1g, h).
3. Cover multiwell plates with the transparent polyethylene foil.
4. Maintain the plants in the growth room at 20 ± 1°C with a 16 h photoperiod and light intensity of 300 μmol m<sup>-2</sup> s<sup>-1</sup>.
5. After 5–6 days, start hardening the plants. Reduce humidity by removing the cover daily for 1 h, and gradually increase the time of acclimation to ex vitro conditions in the successive 4 weeks.
6. Acclimation and hardening of the plants should be performed either in a climatic box that provides temperature and humidity control or in the greenhouse.
7. After 4 weeks, transfer plants to the glasshouse, and after next 4 weeks the plants are ready for transferring into field conditions (Fig. 1i).

### 3.8. Currants and Gooseberries (*Ribes* spp.)

Success of in vitro propagation of *Ribes* spp. depends mainly on the initial explant quality, methods, and time of their excision. Usually, both dormant buds (excised during late winter/early spring) and young green shoot tips (excised in late spring) are used. Explants can originate either from in field-grown stock plantation or from greenhouse-grown plants. However, explants taken

from in field-grown plants have higher contamination rate than those from the greenhouse. As for rooting, recalcitrant genotypes require a two-step rooting procedure for high rooting rate and the formation of well-developed roots. In the first step, the shoots are transferred to a medium containing high concentration of auxin up to the time of root induction (about 10–11 days). Afterwards, shoots are transferred to a medium devoid of plant growth regulators for root elongation.

### **3.9. Preparation and Sterilization of Culture Media**

1. Prepare culture media for initiation, proliferation, and rooting stages according to the formulations mentioned in Table 1 (see Note 9).
2. Add sucrose, vitamins, and plant growth regulators to the required final concentration.
3. Adjust the pH of the media to 5.7–5.8 using 1 M HCl or 1 M KOH.
4. Add 0.6% (w:v) agar.
5. Dispense initiation medium into test tubes, pouring 15 mL medium into each tube, and 25 mL of multiplication or rooting medium into glass jars.
6. Seal the tubes with aluminum foil and put the lids on the jars, sterilize by autoclaving at 121°C for 20–25 min (118 Kpa steam pressure).
7. Store autoclaved media at room temperature in the dark up to 1 month.

### **3.10. Plant Source Material and Surface Sterilization**

1. To establish the culture, different type of explants can be used depending on the season of the year: dormant buds (late winter/early spring) or young green shoot tips (late spring). Explants can be taken from in field-grown plantation or from greenhouse-grown stock plants (see Note 10).
2. When dormant buds are used, excise stem nodal sections with dormant buds and rinse them for 30 min with running tap water.
3. Remove external brown scales from the buds and immerse them in 70% (v:v) alcohol for 1 min, followed by 10–12% (v:v) calcium hypochlorite for 10–12 min (see Note 11).
4. When using 1-year-old green shoot tips, cut the tip one node below the apex and strip off the leaves, leaving the youngest ones. Sterilize 7–9 mm tips with 10–12% (v:v) calcium chlorite for 5 min (see Note 12).
5. Wash explants three times with sterile reverse-osmosis water and keep plant material in small amount of water till the time of dissecting under the stereomicroscope.

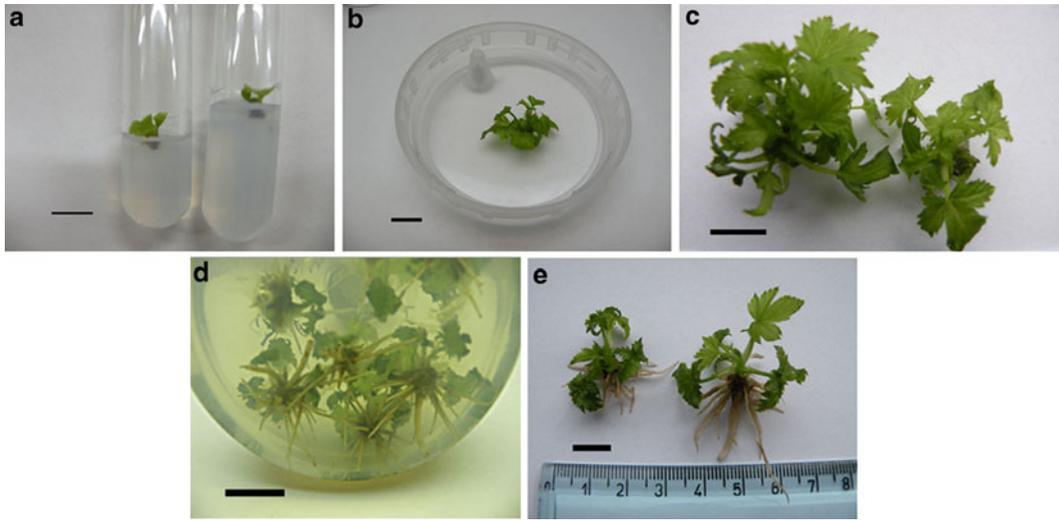


Fig. 2. Proliferation and rooting of currant shoots in culture. (a) Establishment of shoot cultures from dormant buds on MS-based medium (bar=1.0 cm), (b) single shoot on multiplication medium, after 4 weeks of culture (bar=1.0 cm), (c) properly developed shoots after 2 months in culture (bar=1 cm), (d) rooting of shoots on WPM medium with addition of  $2.0 \text{ mg L}^{-1}$  IBA and  $5.0 \text{ mg L}^{-1}$  IAA (bar=1.0 cm), (e) new plantlets, ready to transfer to ex vitro conditions (bar=1.0 cm).

### 3.11. Explant Culture and Maintenance

1. Place the explants on the initiation medium, one per tube, to avoid spreading contamination. Prepare minimum 100 explants per genotype (Fig. 2a).
2. Incubate the cultures at  $25 \pm 2^\circ\text{C}$  under 16 h photoperiod maintained through cool white fluorescent tubes with a photon flux density (PPFD) of  $50 \mu\text{mol m}^{-2} \text{ s}^{-1}$  provided by cool white fluorescent tubes (LF 36 W) at 55–60% relative humidity.

### 3.12. Multiplication of Shoots

1. After stabilization of cultures in 3–4-week time, transfer healthy explants to a fresh medium containing  $1.0 \text{ mg L}^{-1}$  BA and  $0.1 \text{ mg L}^{-1}$  IBA for further shoot multiplication. Subcultures should be done every 4–5-week interval (see Note 13) (Fig. 2b).
2. When chlorosis appears on the regenerated shoots, transfer them onto the medium amended with doubled content of iron (see Note 14).
3. Subculture shoots up to 2.0 cm long onto regeneration medium, and shoots longer than 2.0 cm transfer to the rooting medium.
4. Remove the lower leaves which have a tendency to turn brown.
5. Incubate cultures at  $25/24^\circ\text{C}$  with a 16 h photoperiod,  $70 \mu\text{mol m}^{-2} \text{ s}^{-1}$  (see Note 15).

**3.13. *In Vitro* Rooting of Regenerated Shoots**

1. Transfer 2.0 cm-long shoots to the rooting medium, and insert the base of the shoots into the medium. Culture an average of 6–7 shoots per 330-mL jar (see Note 16) (Fig. 2c).
2. Incubate cultures at 25/24°C, 16 h photoperiod, and light intensity 70  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . After 5–6 weeks, roots develop at the base of the shoots (Fig. 2d).

**3.14. Acclimation of Regenerated Shoots to Ex Vitro Conditions**

1. Remove rooted plantlets from the jars and wash roots carefully with tap water to remove agar.
2. Transfer plants individually to multiwell plates of 5–6 cm, containing universal peat substrate (see Note 17) (Fig. 2e).
3. Water plants thoroughly and protect them from drying, covering the multiwell plates with transparent polyethylene foil.
4. Acclimate the plants in the growth room at  $20 \pm 1^\circ\text{C}$  with a 16 h photoperiod and in proper light intensity, 300  $\mu\text{mol m}^{-2} \text{s}^{-1}$  for 1 month, gradually reducing high humidity by removing the cover daily for 1 h.
5. After 4 weeks, transfer plants to the glasshouse, and finally transfer them to the field conditions (see Note 18).

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**4. Notes**

1. Thornless genotypes are easier to initiate in vitro cultures as compared to thorny genotypes. Shoot tips respond better than the lateral buds in producing multiple shoots.
2. Sterilization time is adjusted depending on the concentration of sterilizing agent and type of explants. Plant materials from field-grown plants are recommended to sterilize twice at 24-h intervals (12).
3. The size of meristem has a great influence on the elimination of virus from tissue. The size 0.2 mm enables to obtain 40–100% virus-free plants, depending on the specific combination virus/cultivar. Meristem explants of higher size are unsuitable for virus elimination, irrespective of the virus and raspberry cultivar.
4. Sometimes a low light intensity reduces leaf chlorosis and shoot dieback, while promoting new shoot and leaf development.
5. At the multiplication stage, it is important to select a proper concentration of BA for the specific genotype. For instance, genotypes more affected by hyperhydricity require a lower concentration of BA (3).
6. When shoots do not elongate on the multiplication medium, transfer them to MS medium devoid of plant growth

regulators; alternatively, a medium added with 0.1–0.2 mg L<sup>-1</sup> GA<sub>3</sub> can be used.

7. The replacement of FeNaEDTA with FeEDDHA is recommended for genotypes requiring more easily assimilated iron ions in the medium.
8. For genotypes recalcitrant to rooting, remove most of the shoots and keep youngest tips, which improves rhizogenesis. Shoots longer than 2.0 cm are easier to handle, their rooting rate is high, and they withstand better stress conditions during acclimation.
9. MS medium is commonly used (22). However, WPM (23) is also recommended.
10. Initial explant size affects the multiplication rate. One-year-old shoot tips give high multiplication rate (10–30%) as compared to shoot tip meristem (5–10%) as initial explants. Larger explants shorten the time of plant material production; however, such a procedure can be used only when the mother-stock plantation is virus-free.
11. For the explants difficult to decontaminate, after standard sterilization procedure, rinse with 1% calcium hypochlorite and keep in this solution up to the time of excision under the stereomicroscope. With the extremely recalcitrant explants (dormant buds), sterilization with 0.1% mercuric chloride for 5 min gives the best results.
12. When using green shoot tips, alcohol treatment should be omitted and concentration of sterilizing agent should be decreased.
13. Genotype and the geographical origin of cultivars can influence shoot multiplication to a large extent. Sometimes higher concentration of BA causes shoot hyperhydricity.
14. Some currant cultivars are susceptible to low concentration of iron components in the media; hence doubling of iron (73.4 mg L<sup>-1</sup> FeNaEDTA) results in healthy, stronger, and green new formed shoots (18).
15. Most often the cool-white fluorescent lamps are used for commercial micropropagation. Yet, the quality of light effects on organogenesis, i.e., shoot initiation is stimulated by blue light, while root initiation by red light.
16. The type of culture medium (solid or liquid) used at the proliferation stage affects the rooting rate. Rooting efficiency is influenced by shoot length; no more than 15% of 15–25 mm long shoots form roots. Thus, longer shoots (35–50 mm long) exhibit a better physiological condition for root formation than the short shoots. The rooting of shoots is inversely proportional to the number of shoots per culture jar with half strength MS medium (16). Sometimes initial treatment of 3 days in the darkness affects positively the rooting of shoots.

17. In acclimation stage, use boxes or bigger containers filled with soil + perlite mixture 2:1 (v:v) (16).
18. Acclimatization of microplants to ex vitro conditions should be performed taking into account the season of the year. High temperature in the mid-summer and physiological changes in plants before dormant phase reduce plant viability.

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# Chapter 12

## Somatic Embryogenesis for Efficient Micropropagation of Guava (*Psidium guajava* L.)

Nasim Akhtar

### Abstract

Guava (*Psidium guajava* L.) is well known for edible fruit, environment friendly pharmaceutical and commercial products for both national and international market. The conventional propagation and in vitro organogenesis do not meet the demand for the good quality planting materials. Somatic embryogenesis for efficient micropropagation of guava (*P. guajava* L.) has been developed to fill up the gap. Somatic embryogenesis and plantlets regeneration are achieved from 10-week post-anthesis zygotic embryo explants by 8-day inductive treatment with different concentrations of 2,4-dichlorophenoxy acetic acid (2,4-D) on MS agar medium containing 5% sucrose. Subsequent development and maturation of somatic embryos occur after 8 days on MS basal medium supplemented with 5% sucrose without plant growth regulator. The process of somatic embryogenesis shows the highest relative efficiency in 8-day treatment of zygotic embryo explants with 1.0 mg L<sup>-1</sup> 2,4-D. High efficiency germination of somatic embryos and plantlet regeneration takes place on half strength semisolid MS medium amended with 3% sucrose within 2 weeks of subculture. Somatic plantlets are grown for additional 2 weeks by subculturing in MS liquid growth medium containing 3% sucrose. Well-grown plantlets from liquid medium have survived very well following 2–4 week hardening process. The protocol of somatic embryogenesis is optimized for high efficiency micropropagation of guava species.

**Key words:** 2, 4-Dichlorophenoxy acetic acid, Artificial seeds, Fruit tree, Guava, Micropropagation, *Psidium guajava*, Somatic embryogenesis, Zygotic embryo

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### 1. Introduction

Guava is a rich source of proteins, carbohydrates, fats, vitamins, minerals, fragrance, and flavor and is within the reach of a common man and other poorer sections of the society worldwide. Probably, it occupies the prime position among the fruit crops and called “poor man apple.” Guava has antidiarrheal, antibacterial, antiamoebic,

and antispasmodic activities. High concentrations of several vitamins, dietary fiber, carotenoids, lectins, saponins, tannins, phenols, triterpenes, and flavonoids altogether make guava therapeutically an important fruit. Guava fruits lower blood cholesterol, triglycerides, hypertension, and some cardiac problems. Due to high quercetin contents, guava leaves are used to develop a phyto-medicine for diarrhea. Ascorbic acid in guava fruit far exceeds that in citrus. It contains almost five times as much Vitamin C as orange. Guava is largely consumed as a fresh fruit and in processed forms such as jelly making.

Common guava (*Psidium guajava* L.; Family Myrtaceae) is diploid ( $2n=22$ ), but exist as triploid ( $2n=3x=33$ ) in some natural and artificial forms with seedless fruits. It is endemic to tropical America, but has been thoroughly naturalized throughout the tropics and subtropics. Guava flowers are self- and natural cross-pollinated for the 30–35% (1), produce three crops in 1 year, and in a mild tropical climate the tree can flower and fruit throughout the year. Guava cultivars display a wide diversity of tree-size, bearing habit and yield, as well as fruit shape, size, quality, and ripening season (1, 2). Seedless triploid guava and other cultivars have been conventionally propagated through budding inarching, or approach grafting to maintain its clonal populations (2).

Guava has been successfully micropropagated through the nodal explants and regenerated adventitious shoots from leaves of already micropropagated in vitro-grown plantlets (3–7). Subsequently, clonal propagation of guava from seedling and grafted plants has been accomplished (8). Earlier reports indicate low regeneration rate which has been found due to high phenolic exudation and media browning; the drying of explants prior to inoculation on the media prevents browning. As a result, the rapid multiplication of guava seedlings through in vitro shoot tip culture has been over 95% without any problem of phenolics or necrosis of the explants (9).

Somatic embryogenesis from zygotic embryo cultures of guava is reported by several researchers (10). We have made comprehensive studies on the induction of somatic embryogenesis and production of artificial seeds for micropropagation and its applications to improve guava and other tropical and subtropical fruit species (10–13). Further improvement in somatic embryogenesis has been reported with varying success and is optimized for various cultivars of guava at several locations in India (10, 14–18). This chapter describes stepwise protocol of somatic embryogenesis for efficient micropropagation of different guava cultivars.

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## 2. Materials

### **2.1. Culture Medium and Stock Solutions**

1. The medium is based on the original formulation of Murashige and Skoog (MS) (19).
2. The composition of MS medium formulation and the amount of each component required for preparation of specific concentrations and volume of stock solutions are given in Table 1.
3. Double distilled water.
4. Measuring cylinders.
5. Conical flasks.
6. Pipettes.
7. Screw capped reagent bottles.
8. Culture tubes or culture bottles.
9. Tissue culture facilities: digital balances, precalibrated digital pH meter, magnetic stirrer and bars, hot air oven, hot plate, autoclave.

### **2.2. Surface Sterilization of Explants Material**

1. Tap water.
2. 80% (v/v) ethanol prepared with sterile double distilled water.
3. Autoclaved 0.05% HgCl<sub>2</sub> solution.
4. Autoclaved double distilled water; 150 mL aliquots in 250 mL cotton plugged conical flasks.
5. Autoclaved small cotton rolls.
6. Empty plugged conical flasks.
7. Empty plugged culture tubes.
8. Petri dishes.
9. Glass slides.
10. Autoclaved culture tubes.
11. 100 mL glass measuring cylinder.
12. Glass beakers and glass pipettes of different capacity.
13. Cotton plugs.
14. Tissue culture facilities—Instruments (scissors, scalpel, forceps, needle), laminar flow bench, culture room, dissecting microscope, culture tube-stand, wire baskets, glass bead sterilizer.

### **2.3. Encapsulation of Somatic Embryos**

1. Autoclaved 2% (w/v) sodium alginate (Sigma, USA) gel in double distilled water.
2. 50 mL aliquot of 100 mM CaCl<sub>2</sub> solution in 150 mL conical flasks.

**Table 1**  
**Composition, concentration, and amounts of various nutrient salts used for the preparation of different volumes of stock solutions for Murashige and Skoog (MS, (19)) medium**

Ingredients of stock solutions	Amount present in medium (mg L <sup>-1</sup> )	Quantity (mg) required to prepare specific volume of different stock solution (mL)				
		1,000	500	250	100	50
Stock solution-I (20×) (macronutrients)						
NH <sub>4</sub> NO <sub>3</sub>	1,650	33,000	16,500	8,250	3,300	1,650
KNO <sub>3</sub>	1,900	38,000	19,000	9,500	3,800	1,900
CaCl <sub>2</sub> ·2H <sub>2</sub> O	440	8,800	4,400	2,200	880	440
MgSO <sub>4</sub> ·7H <sub>2</sub> O	370	7,400	3,700	1,800	740	370
KH <sub>2</sub> PO <sub>4</sub>	170	3,400	1,700	850	340	170
Stock solution-II (200×) (micronutrients)						
KI	0.835	166	83	41.5	16.6	8.3
H <sub>3</sub> BO <sub>3</sub>	6.2	1,240	620	310	124	62
MnSO <sub>4</sub> ·4H <sub>2</sub> O	22.3	4,460	2,230	1,115	446	223
ZnSO <sub>4</sub> ·7H <sub>2</sub> O	8.6	1,720	860	430	172	86
Na <sub>2</sub> MoO <sub>4</sub> ·2H <sub>2</sub> O	0.25	50	25	12.5	5	2.5
CuSO <sub>4</sub> ·5H <sub>2</sub> O	0.025	5	2.5	1.25	0.5	0.25
CoCl <sub>2</sub> ·6H <sub>2</sub> O	0.025	5	2.5	1.25	0.5	0.25
Stock solution-III (200×) (iron source)						
FeSO <sub>4</sub> ·7H <sub>2</sub> O	27.8	5,560	2,780	1,390	556	278
Na <sub>2</sub> -FDTA·2H <sub>2</sub> O	37.3	7,460	3,730	1,865	746	373
Stock solution-IV (200×) (organic nutrients)						
Inositol	100	20,000	10,000	5,000	2,000	1,000
Nicotinic acid	0.5	100	50	25	10	5
Pyridoxine HCl	0.5	100	50	25	10	5
Thiamin HCl	0.1	20	10	5	2	1
Glycine	2	400	200	100	40	20

3. Double distilled water.
4. Glass burette with stand.
5. Needles.
6. Dissecting microscope.
7. Glass slides.

#### **2.4. Acclimatization of Plantlets**

1. Tap water.
2. Small plastic pots (9 cm diameter), medium and large size plastic bags or earthen pots.
3. Potting medium sand: soil: compost (5:1:2 and 1:2:3 by weight).
4. Sprayer or fogging instruments.

### 3. Methods

#### 3.1. Preparation of Stock Solutions

1. Prepare stock solution I: 20× concentration in 1,000 mL distilled water.
2. Prepare stock solution II, III, and IV: 200× concentration in 100 mL distilled water.
3. Measure 1,000 or 100 mL double distilled water using volumetric flask.
4. Weigh appropriate amount of chemicals (Table 1) for preparing 20× stock solution I: dissolve sequentially each of them in small amount of water and finally raise volume to 1,000 mL by adding doubled distilled water; transfer into 1,000 mL screw cap reagent bottle, label as solution I (20×).
5. Similarly, prepare stock solutions II and IV, 200× concentration (Table 1), and store in 100 mL screw cap reagent bottles, properly label as stock solution II (200×) and stock solution IV (200×).
6. Chelated iron solution preparation: prepare 100 mL of 200× stock solution III by weighing the required amount (Table 1) of  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  and  $\text{Na-EDTA} \cdot 2\text{H}_2\text{O}$  separately; add 40 mL double distilled water in each of them in two different 250 mL conical flask; dissolve by heating over hot plate; mix both solutions and raise the final volume to 100 mL by adding doubled distilled water; transfer into 100 mL screw capped reagent bottle; label as stock solution III (200×).
7. Store all stock solution at 4°C in a refrigerator.

#### 3.2. Preparation of Stock Solutions of Growth Regulators

1. Weigh 5 or 10 mg 2,4-dichlorophenoxy acetic acid (2,4-D) powder, transfer in a clean dried small test tube, and dissolve in 0.1–0.2 mL 80% ethanol (see Notes 1 and 2).
2. Maintain the final volume with double distilled water to get the final concentration of 1 or 2 mg mL<sup>-1</sup> stock solution.

#### 3.3. Preparation of Culture Medium

1. To prepare 1 L MS basal medium, measure 50 mL stock-I (20×) and 5 mL stock-II, III, and IV (each 200×) and add one by one to 900 mL double distilled water and raise the final volume to 1 L (see Note 3).
2. Transfer the medium to 1 L beaker and check the initial pH. It should be around  $4.2 \pm 0.05$  after mixing all the four stock solutions.
3. Raise pH of the medium to  $6.2 \pm 0.05$  with the addition of 1 M NaOH.
4. Leave the medium under continuous stirring over a magnetic stirrer for 1–2 h.

5. After about 1 h the pH of the medium stabilizes at  $5.8 \pm 0.05$ , if not can be adjusted with 0.1 M HCl (see Note 4).
6. For induction and development media, weigh 50 g sucrose and dissolve in 1,000 mL (5%w/v) medium; for germination and plantlets growth media dissolve 30 g sucrose in 1,000 mL (3%w/v) medium (see Subheading 3.5 for media modifications).
7. To prepare semisolid medium weigh 8–10 g agar (0.8–1.0% w/v) and add to the above 1 L medium.
8. Melt agar by gentle heating over a hot plate with continuous stirring.
9. Use melted agar to modify the culture medium according to the need and stages of somatic embryogenesis.

### **3.4. Culture Vessel**

1. Most of the stages of somatic embryogenesis are carried out in culture tubes (25×150 mm), plugged with muslin cloth wrapped cotton plugs for the proper aeration of the growing cultures and to minimize culture contamination.
2. Culture tubes plugged with polycarbonate caps are also used, producing a slight variation in the embryogenic frequency is expected.
3. Use 150 or 250 mL capacity conical flasks for plantlet growth.

### **3.5. Media Types and Modifications**

#### *3.5.1. Induction Medium*

1. Prepare full strength semisolid MS medium supplemented with 5% (w/v) sucrose and  $1.0 \text{ mg L}^{-1}$  2,4-D for the induction of somatic embryogenesis.
2. Dispense 8–10 mL induction medium amended with 2,4-D to each culture tube.
3. Plug the culture tubes with muslin cloth wrapped cotton plugs and autoclave.
4. Modify culture media with 0.1, 0.5, 1.5, and  $2.0 \text{ mg L}^{-1}$  2,4-D when using zygotic embryo explants of various cultivars from different agroclimatic zones.

#### *3.5.2. Development and Maturation Medium*

1. Prepare full strength agar-solidified MS basal medium supplemented with 5% (w/v) sucrose without any plant growth regulator (PGR).
2. Dispense 10–12 mL of medium into culture tubes and plug the tubes with muslin cloth wrapped cotton plugs; sterilize by autoclaving.
3. Use the development medium, free of PGR, for subculture of explants following 8 days of inductive treatment with 2,4-D.

**3.5.3. Germination Medium**

1. Prepare half strength agar-solidified MS basal medium supplemented with 3% (w/v) sucrose without PGR for the germination of somatic embryo and encapsulated seeds (see Subheading 3.9).
2. Dispense 12–15 mL of the medium in each culture tube, plug the tubes with muslin cloth wrapped cotton plugs and autoclave.
3. For germination of encapsulated seeds, autoclave medium in conical flasks and pour 20–25 mL medium into sterile Petri dishes.

**3.5.4. Growth Medium**

1. Prepare full strength MS liquid suspension medium as mentioned in Subheading 3.3 for the initial growth of plantlet prior to the soil transfer and hardening.
2. Supplement this medium with 3% (w/v) sucrose devoid of PGR.
3. Dispense 25–30 mL full strength MS liquid suspension medium to each 150 or 250 mL conical flasks for plantlet growth.
4. Plug the culture vessels with cotton plug and sterilize the medium by autoclaving.

**3.6. Stock Solution of Mercuric Chloride**

1. Weigh 50 mg  $\text{HgCl}_2$  and dissolve in 100 mL double distilled water in 250 mL conical flask to get 0.05% concentration (see Notes 5 and 6).

**3.7. Sterilization**

1. Sterilize different media,  $\text{HgCl}_2$  solution, double distilled water, and all other instruments by autoclaving at 121°C for 15 min at 1.1 kg  $\text{cm}^{-2}$  pressure (see Note 7).

**3.8. Plant and Explants****3.8.1. Selection of Cultivars and Plants**

1. Select a 10–15 year old tree of an elite high-yielding guava variety from the field.
2. Collect fruits from the selected guava tree and use them to excise zygotic embryo explants.
3. Observe the development of flower buds systematically in each growing season with regard to their appearance, growth periods, and maturity.
4. The full grown or the half-open flower buds (about 14–18 days from their appearance), are tagged and bagged to ensure close/open pollination; time of explant collection is regarded as the day zero of anthesis and fertilization.

**3.8.2. Collection of Fruits and Preparation of Explant Material**

1. Collect guava fruits ranging from 2.5 to 4.0 cm diameter after 10 weeks of anthesis.
2. Wash the fruits thoroughly under running tap water for about 10–15 min.

3. Remove the hard green exo- and greenish white mesocarp with the help of a sharp knife or scalpel and divide the central pulp with seeds into 4–6 vertical lobes.
4. Wash the central pulp lobes under running tap water for 15–20 min.
5. Disinfect the central pulp with seeds by treating with a mild antiseptic (e.g., 2% Cetavlon (v/v); ICI Ltd., India) with 2–4 drops of Tween-X (Hi-media, India) in 50 mL tap water for 15 min in 150 or 250 mL conical flasks.
6. Wash the material under running tap water for further 10–15 min to remove the traces of surfactants.
7. Rinse in 80% (v/v) ethanol for 30–60 s followed by treating the pulp lobes with an autoclaved 0.05% HgCl<sub>2</sub> solution (w/v) for about 15–17 min by rigorous shaking in a sterilized 250 mL conical flask.
8. Rinse the pulp lobes 4–5 times with sterile double distilled water to remove traces of sterilant.

### 3.8.3. Dissection of Zygotic Embryo Explants

1. Put one of the pulp lobes along with seeds in a 96 mm diameter sterilized Petri dish, containing 15–20 mL sterile double distilled water.
2. Pick up guava seeds from the sterilized pulp lobes using forceps and scalpel; place on a microscopic slide for dissection.
3. Hold the seed with a forceps in left hand over a microscopic slide and remove a small piece of longer arm with the help of a fine and pointed scalpel.
4. Turn the seed in opposite orientation and similarly remove a small piece of shorter arm, preferably by making a horizontal cut.
5. Insert a blunt needle either through the longer or the shorter arm side, so that embryo comes out at the other end.
6. Inoculate the zygotic embryo (Fig. 1a) on induction medium, modified with 2,4-D, in a test-tubes.
7. Incubate the culture for 8 days for induction of somatic embryogenesis (see Note 8).
8. Transfer zygotic embryos from induction to development medium after 8 days of inductive treatment.
9. Zygotic embryos from cultivars of different agroclimatic zones are tested with 0.1, 0.5, 1.0, 1.5, and 2.0 mg L<sup>-1</sup> 2,4-D containing medium for optimization of somatic embryogenesis (see Note 9).

### **3.9. Encapsulation of Somatic Embryos**

1. Weigh 2 g sodium alginate and dissolve in 100 mL of either double distilled water or quarter strength MS basal medium to prepare 2% alginate gel for encapsulation of somatic embryos.
2. Optionally supplement the gel with 0.1 or 1.0% sucrose.
3. Prepare 100 mM calcium chloride solution in 1,000 mL double distilled water and dispense 30–50 mL aliquots in 150 or 250 mL conical flask.
4. Take 10 set of 100 mL double distilled water in 150 mL conical flasks, plug with muslin cloth wrapped cotton plugs.
5. All the chemicals including sodium alginate, calcium chloride, distilled water, and glasswares are sterilized by autoclaving; encapsulation is carried under laminar air-flow hood.
6. Separate various stages of somatic embryos from zygotic embryo explants after 10 weeks of culture initiation with the help of fine needles under dissecting microscope (10–12, 18).
7. Mix separately two types, i.e., elongated torpedo (ET) and short torpedo (ST) stages somatic embryos (10–12, 18), in 2% sodium alginate gel.
8. Transfer the alginate and embryo mixture to burette (wide tip) stand vertically and drop alginate-coated somatic embryos to calcium chloride solution.
9. Alternatively, somatic embryo is inserted into alginate gel flowing from a vertically fixed burette just before dropping into CaCl<sub>2</sub> solution.
10. The alginate drop takes the shape of spherical beads (4–6 mm size) immediately after falling into the calcium chloride solution (Fig. 1g).
11. Pour off the calcium chloride solution after 30–45 min when the beads turn milkish white and become slightly harder; rinse the encapsulated embryos 4–5 times with sterile double distilled water.
12. Subculture artificial seeds immediately (or after storage) on germination medium (Fig. 1h).

### **3.10. Culture Environment**

1. Incubate all cultures at  $25 \pm 2^\circ\text{C}$ , 60–65% relative humidity, 16 h photoperiod at a photon flux density (PFD) of  $50\text{--}70 \mu\text{mol m}^{-2} \text{s}^{-1}$  provided by two 40 W white fluorescent tubes from a distance of 30–35 cm in each shelf.
2. The light intensity at an elevated PFD of  $70\text{--}80 \mu\text{mol m}^{-2} \text{s}^{-1}$  is provided by three to four 40 W white fluorescent tubes from a distance of 30–35 cm in each shelf during germination and growth phase of the process.

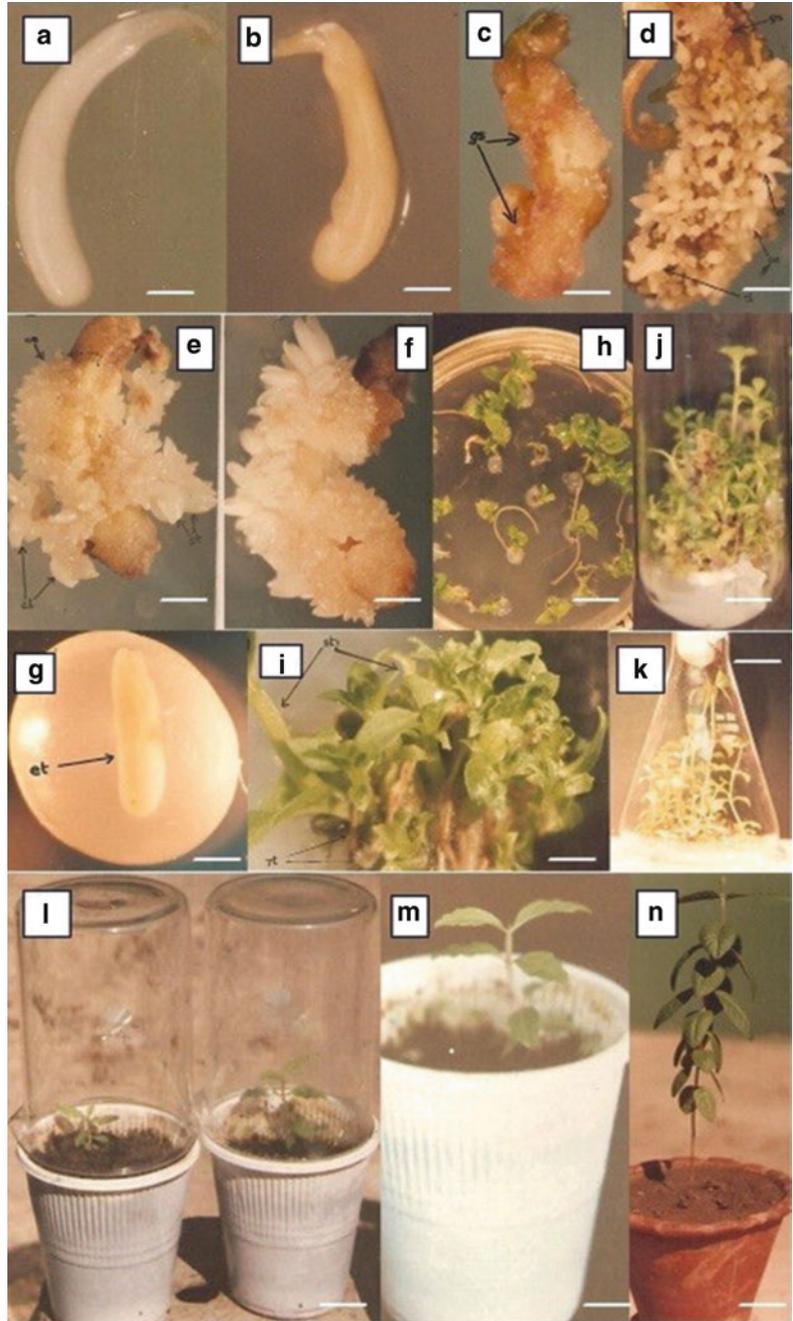


Fig. 1. Regeneration of plantlet from somatic embryos and artificial seeds of guava (*Psidium guajava* L.); (a) ten-week post-anthesis zygotic embryo used as explants source (Bar=0.65 mm), (b) zygotic embryo explant on 2,4-D containing induction medium after 8 days of culture initiation (Bar=0.75 mm), (c) zygotic embryo after 3 weeks of culture initiation showing development of numerous globular stage somatic embryos (Bar=0.81 mm), (d) early stages in somatic embryo development from zygotic embryo explant after 4 weeks of culture initiation (Bar=0.72 mm), (e) development and growth of somatic embryos from zygotic embryo explants after 5 weeks of culture initiation (Bar=1.25 mm), (f) somatic embryos at advanced stages of development from zygotic

### 3.11. Potting Mixture

1. Weigh and mix well all the three components of potting mix, viz., sand: soil: compost in the ratio of 5:1:2 (w/w/w).
2. Fill plastic glass pots (9 cm diameter) or multichannel plastic growth pots (30 × 30 cm) specific for plantlet transplantation.
3. Water the pots thoroughly in order to wet the potting mix uniformly.
4. Prepare similarly the large plastic glass (250 mL) or small polyethylene bags with sand: soil: compost in the ratio of 1:2:3 (w/w/w).
5. Prepare similarly medium size polyethylene transfer bags with normal garden soil used in nursery.

### 3.12. Somatic Embryogenesis and Regeneration of Plantlets

#### 3.12.1. Stages in Somatic Embryogenesis and Plantlets Regeneration

##### Induction of Somatic Embryogenesis

The process of somatic embryogenesis for micropropagation of guava is described in various different stages, viz., induction of somatic embryogenesis, development and maturation of somatic embryos, germination of somatic embryos, growth of plantlets, and acclimatization of plantlets to ex vitro condition (10–12, 18).

1. Isolate zygotic embryo explants by dissecting seeds from 10-week post-anthesis fruits; embryos are curved, translucent watery white, mostly with 3–5 mm long embryonic axis, and 3–5 times longer hypocotyl than the cotyledons (Fig. 1a).
2. Zygotic embryo explants are cultured for 8 days on full strength agar-solidified MS basal medium supplemented with 5% sucrose and 1.0 mg L<sup>-1</sup> 2,4-D for the induction of somatic embryogenesis (Fig. 1b).

←

Fig. 1. (continued) embryo explants after 6 weeks of culture initiation (Bar = 1.5 mm), (g) an artificial seed encapsulating an elongated torpedo stage somatic embryo (10 weeks after culture initiation) in sodium alginate gel (Bar = 0.63 mm), (h) germination of artificial seeds (encapsulating 10-week-old elongated torpedo stage somatic embryos) on half strength MS agar-solidified medium with 3% sucrose, showing the development of shoots and roots (Bar = 13.5 mm), (i) germination of different stages of somatic embryos in the second week of subculture to half strength MS agar-solidified basal medium with 3% sucrose (Bar = 2.75 mm), (j) development of somatic plantlets after 2 weeks of subculture on half strength MS agar-solidified basal medium with 3% sucrose (Bar = 7.62 mm), (k) growth of plantlets after 2 weeks of subculture in full strength MS liquid basal medium added with 3% sucrose (Bar = 21 mm), (l) somatic plantlets in the process of acclimatization (Bar = 18 mm), (m) a somatic plantlet after 2 weeks of hardening process (Bar = 12 mm), (n) a guava plant regenerated through somatic embryogenesis after 6 weeks of soil transfer and hardening (Bar = 150 mm) (*ct* cotyledondary stage somatic embryos; *et* elongated torpedo stage somatic embryos; *gs* globular somatic embryos; *h* heart-shaped somatic embryos; *rt* root; *sh* shoots; *st* short torpedo stage).

Development  
and Maturation  
of Somatic Embryos

1. Development and maturation of somatic embryos (10–12, 18) is done on the development medium having full strength agar-solidified MS basal medium containing 5% sucrose but free of PGR (Fig. 1c–f) (see Notes 10–21).
2. Both induction and developmental phases of somatic embryogenesis are carried out under the similar culture environment (see Subheading 3.10 and Table 2).

Germination  
of Somatic Embryos

1. Subculture the entire bunch of somatic embryos along with the zygotic embryo explants after 10 weeks of culture initiation to a fresh embryo germination medium consisting of half strength agar-solidified MS basal salts and supplemented with 3% (w/v) sucrose.
2. Incubate culture under high PFD (70–80  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) (see Subheading 3.10).
3. Somatic embryos subcultured on germination medium will turn light green to green irrespective of the developmental stages, indicating germination within 2–3 days of transfer.
4. Most of the somatic embryos show the emergence of root, shoot, and leaves within the first week of subculture.
5. Germinating somatic embryos continue to grow into plantlets during the second week of subculture (see Note 22).
6. At the end of second week, most of the plantlets develop well-grown roots (0.5–2.0 cm), shoots (0.5–5.0 cm), and leaves (medium size) (Fig. 1i, j).
7. There is no significant growth of plantlets during third weeks of subculture due to nutrient depletion.

Germination  
of Artificial Seeds

1. Subculture artificial seeds in Petri dishes using tap water, or on sterile sands soil mixture or on half strength agar-solidified MS basal medium with 3% (w/v) sucrose.
2. Incubate artificial seed cultures in culture room under high PFD (70–80  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) (see Subheading 3.10).
3. Germination of artificial seeds takes place by emergence of root and shoot from the alginate matrix within 1 week of subculture.
4. Growth of proper roots, shoots, and leaves takes place only on half strength agar-solidified MS germination medium with 3% (w/v) sucrose (Fig. 1h).

Growth of the Plantlets

1. Subculture the plantlets regenerated from somatic embryos or artificial seeds after 2 weeks on full strength MS liquid growth medium containing 3% (w/v) sucrose.

**Table 2**  
**Effect of 2,4-dichlorophenoxy acetic acid on induction of somatic embryogenesis from immature zygotic embryo explants of guava (*Psidium guajava* L.)**

Conc. of 2,4-D (mg L <sup>-1</sup> )	Frequency of embryogenesis		Intensity of embryogenesis		Frequency of various stages of somatic embryos			Efficiency of embryogenesis
	FE (%)	IE (ANEPC)	ET (%)	ST (%)	CHG (%)	EE (relative)		
1	0.000	0.000	0.000	0.000	0.000	0.000	0.000	
2	6.94±2.41	15.33±2.52	23.77±3.40	49.12±3.65	27.11±2.67	0.75±0.19		
3	27.78±4.81	19.38±1.84	21.41±2.93	39.48±3.70	39.11±6.60	3.24±0.38		
4	38.89±4.82	24.51±3.82	23.28±5.77	41.11±2.94	35.61±8.36	6.05±0.65		
5	47.22±4.81	36.20±7.46	19.28±1.86	39.53±3.52	41.19±5.03	10.07±2.54		
6	66.67±8.34	109.33±31.21	19.33±2.75	40.17±2.15	40.50±4.87	43.43±14.20		
7	67.23±5.88	191.31±34.17	16.43±3.53	37.43±4.60	46.14±5.60	68.97±15.02		
8	85.58±7.29	360.04±81.87	16.17±3.53	36.95±3.40	46.89±4.14	167.72±56.85		
9	46.67±7.45	91.47±8.77	4.10±0.86	18.17±4.13	77.73±4.81	9.88±4.55		
10	16.67±5.89	37.48±6.53	0.000	8.63±2.04	91.37±2.04	0.50±0.10		
11	0.000	0.000	0.000	0.000	0.000	0.000		

Ten-week-old zygotic embryos of guava were treated for 8 days with different concentrations of 2,4-D in full strength MS medium added with 5% sucrose, followed by subculturing to full strength MS development medium containing 5% sucrose without any plant growth regulator. Embryogenic responses were noted after 10 weeks of culture initiation

2,4-D 2,4-dichlorophenoxy acetic acid; ANEPC average number of embryos per culture; CHG cotyledonary, heart, globular stage somatic embryos; ET elongated torpedo stage somatic embryos; MS Murashige and Skoog's medium (19); ST short torpedo stage somatic embryos. Figures represent mean value±SD

2. Maintain cultures for an extended growth period of 2 weeks in culture room at PFD of 70–80  $\mu\text{mol m}^{-2} \text{s}^{-1}$  (see Subheading 3.10).
3. All the plantlets grow well in growth medium and attain full height of culture vessel within 2 weeks of subculture (Fig. 1k). (10–12, 18).

Acclimatization  
of Regenerated Plantlets  
to Ex Vitro Conditions

1. In vitro-grown plantlets are taken from the growth medium to plant transfer area.
2. Wash plantlets gently under slow running tap water for about 15–30 min to remove nutrient salts and sucrose adhering to root surface without causing any damage.
3. Plantlets from liquid growth medium initially require a more porous potting mixture, i.e., 5:1:2 (w/w/w) of sand: soil: compost.
4. Transfer plantlets either singly or in a group of two in each pot without any damage to the roots.
5. Keep the plantlets covered with glass beakers or with transparent polyethylene cover for the first 2–4 days and maintain high humidity during transplantation (Fig. 1l).
6. Remove cover periodically for progressively increasing time periods and whenever the cover looks foggy or leaves appear water soaked or turgid (Fig. 1m).
7. Cover the plantlets whenever leaves become flaccid, water the pots if necessary.
8. Grow the plantlets for 1–2 weeks in a growth room fitted with fogging (or humidifier) and large exhaust fans (or dehumidifier), temperature, and photoperiod control system, till a good root system, shoot and leaf development is observed.
9. Transfer the plants to a potting mix with a ratio 1:2:3 (w/w/w) of sand: soil: and compost in medium size polyethylene bags or earthen pots after 2 weeks of growth (10, 11, 18).
10. Keep the plantlets in a shady place of the glasshouse equipped with fogging (or humidifier) and large exhaust fans (or dehumidifier), temperature and photoperiod control system.
11. Maintain slightly higher relative humidity during initial phase of hardening process.
12. Transfer the plantlets in large polyethylene bags with garden soil and store in the growth area of nursery covered with growth net (Fig. 1n).
13. Water the potted plants regularly and mulch the upper layer of potting mix occasionally.

14. The somatic plants are ready for the field transfer following 4–6 weeks of acclimatization (see Note 23).
15. Monitor the growth performance of plants under the field condition.
16. Maintain a record of the developmental history and performance of plants for quality assurance.

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## 4. Notes

1. For the induction of somatic embryogenesis, 2,4-D is the best for high frequency of normal and convertible somatic embryo production.
2. Always prepare a fresh stock of 2,4-D solution.
3. Avoid direct mixing of all the stock solutions, as they form complexes and reduce embryogenic frequency.
4. Limit the use of HCl to adjust pH of the medium as it reduces the pH during autoclaving and hinders agar solidification.
5. Alternatively, sodium hypochlorite or any other source of chlorine can be used at recommended concentration and period of treatment.
6. Dispose the used  $\text{HgCl}_2$  solution following the local recommended procedure.
7. A glass bead sterilizer is used to sterilize the forceps, scalpels, and other stainless steel instruments for dissection and inoculation of zygotic embryo explants.
8. Induction treatment for less than 8 days is insufficient for high efficiency plantlet production.
9. Select appropriate developmental stage of zygotic embryos to be used as explants, according to environmental or seasonal variations of different agroclimatic zones.
10. Somatic embryos are visible as small watery white translucent globules under stereomicroscope usually after or at the end of the third week of culture initiation (Fig. 1c).
11. In the fourth week of culture, several new globular somatic embryos appear while the older ones increase in size and proceed to the early and late heart-shaped embryos with simultaneous change in the color from watery white transparent to translucent milkish (Fig. 1d).
12. In the fifth and sixth week of culture initiation, some new globular somatic embryos appear while the elder ones continue to grow as cotyledonary stage, short torpedo-shaped, and elongated torpedo-shaped embryos (Fig. 1e, f).

13. The number of somatic embryos is varying considerably, ranging from a few to even 150–250 embryos per zygotic embryo explant, in the presence of  $1.0 \text{ mg L}^{-1}$  2,4-D (see Table 2, Fig. 1c–f).
14. Somatic embryos follow a normal developmental and maturation pathway; the frequency of anomalous development and secondary embryogenesis is negligible or very low when following the present protocol.
15. Development of somatic embryo is asynchronous, showing 4–5 discernible stages and more in 5 week old cultures (Fig. 1c–f) (10–12, 18).
16. Depending upon their sizes and post-developmental response, i.e., response of somatic embryos during maturation, germination, and encapsulation, they are grouped into three categories (10–12, 18).
17. The most preferred somatic embryos (longer than 1.5 mm) are called elongated torpedo stage (ET); slightly less preferred (ranging from 1.0 to 1.5 mm in size) are termed short torpedo stage (ST); the least preferred somatic embryos at cotyledonary, heart, and globular stages of development (smaller than 1.0 mm) are grouped into a third category (CHG stage).
18. Somatic embryos during their developmental progression from globular to torpedo stage mature simultaneously onto the same development medium.
19. After 8 weeks of culture initiation, most of the somatic embryos are hard, solid milky white, irrespective of their morphology or developmental stage.
20. Of the total number of somatic embryos, about 15–18% develop into elongated torpedo (ET) stage; 33–35% develop into short torpedo (ST) stage, mature properly, and are convertible into normal plantlets after transfer to the germination medium (Table 2).
21. Almost all the somatic embryos remain in the same state without any further growth if they are maintained on the same development medium even up to 14 weeks of culture initiation.
22. Of the total numbers of somatic embryos, 90–95% of ET stage and 70–85% of ST stage are converted into normal plantlets during 2 weeks of subculture into germination medium.
23. Of the total number of plants from the growth medium, about 80–90% of them survive well after proper hardening and acclimatization process. The plants perform well in the field and set fruits within a period of 1.5 year.

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## Micropropagation of Chokeberry by In Vitro Axillary Shoot Proliferation

Wojciech Litwińczuk

### Abstract

The black chokeberry—aronia (*Aronia melanocarpa* Elliot) is a shrub native to North America although nowadays well known in Eastern Europe. The fruits are regarded as the richest source of antioxidant phytonutrients among fruit crops and vegetables. Chokeberries can be easily propagated by seeds but this method is not recommended. Micropropagation is far more efficient than other conventional cloning methods like layering or softwood cuttings. *Aronia* clones are propagated in vitro through four- or three-stage method based on subculturing of shoot explants. The double diluted MS or full strength MS medium with elevated 50% Ca<sup>2+</sup> and Mg<sup>2+</sup> content are used in the initiation and proliferation chokeberry in vitro cultures, respectively. They are supplemented with 0.5–1.0 mg LBA, and 0.05 mg LIBA. The double-phase medium is recommended in the last passage before shoot rooting. The regenerated shoots could be rooted both in vitro on double diluted MS with 0.05 mg L<sup>-1</sup> IBA or in vivo in peat and perlite substrate and subsequently grown in the greenhouse.

**Key words:** *Aronia*, Axillary shoots, Nodal explants, Small fruits, Tissue culture

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### 1. Introduction

The black chokeberry—aronia (*Aronia melanocarpa* Elliot) is a shrub native to North America although nowadays well-known, cultivated and utilized in Poland, Russia, and other countries of Eastern Europe. The fruits are rich in macro- and micronutrients (Ca, Fe, Mo, Mn, Cu, B, I, Co), vitamins (P, C, B<sub>2</sub>, B<sub>6</sub>, PP, E, proA), carbohydrates, cellulose, pectins, anthocyanins, proanthocyanidins, and catechins. The antioxidant effect of aronia fruits is much higher than cranberries, elderberry and blackberries, and even blackcurrants. Black chokeberry fruits are used in food industry and pharmacy. Chokeberries can be easily propagated by seeds. This method is not recommended because of late bearing of plants, vigorous and non-uniform growth, and plants being unsuitable for

mechanical harvest. Black chokeberry is comparatively a young crop and only few cultivars or breeding clones are known and grown. As micropropagation is far more efficient than other conventional cloning methods, it should improve breeding and rapid propagation of new, valuable strains of *Aronia*. The studies on black chokeberry micropropagation were so far carried out by several authors (1–7). There are few reports concerning other applications of in vitro cultures: cryopreservation (8), and induction of colchiploids (9).

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## 2. Materials

### 2.1. Preculture (Preparation of Source Material)

1. Young progeny of selected, well-evaluated, and bred plants, in plastic pots (ca 2 L volume) filled with universal potting medium consisting compost or healthy shoots of aforementioned plants (see Note 1).
2. “Forcing medium” (Table 1).
3. Fungicides (Bayleton 5 WP 0.1%, Topsin M 0.15%)

### 2.2. Surface Sterilization of Source Material

1. Tap water.
2. Ethanol 70% (v:v).
3. Commercial bleach solution (e.g., “ACE” bleach; 5% (v:v) NaOCl), diluted 1:5 (v:v) with tap water.
4. 150 mL aliquots of autoclaved reverse-osmosis (or distilled) water in 300 mL screw capped jars.
5. Laboratory shaker or ultrasonic washer.
6. Shoots of parental plant as a source of explants.

### 2.3. Tissue Culture Facilities and Culture Media

1. Labware (autoclave, magnetic stirrer with heating, dry-heat sterilizer, microwave oven, pH meter, refrigerator, horizontal laminar flow bench).
2. Instruments (scalpels, forceps, glass bead sterilizers, or gas burners).
3. 1 M HCl and 1 M NaOH.
4. Sterile Petri dishes (100 × 15 mm) or sterile paper sheets (kept at 160°C for minimum 2 h) to prepare explants.
5. Sterile graduated (25 mL) pipettes with pumps, autoclavable and adjustable (1–5 mL) single-channel pipettes or other dispensers of liquid medium to make double-phase medium.
6. Media based on the formulation of MS (10) for (a) parental shoot forcing, (b) culture initiation, (c) shoot regeneration from stem nodal explants, (d) shoot elongation, and (e) root

**Table 1**  
**Culture media based on the formulation of Murashige and Skoog (MS; (10))**  
**used for micropropagation of black chokeberry**

Stages	Shade-forcing parental shoots	Initiation	Proliferation	Rooting in vitro
Macronutrients	25% MS	50% MS		50% MS
N salts			100% MS	
K salts			100% MS	
P salts			100% MS	
Ca salts			150% MS	
Mg salts			150% MS	
Micronutrients (without Fe salts)	25% MS	50% MS	100% MS	50% MS
NaFeEDTA (mg L <sup>-1</sup> )	9.1	18.4	55.1	18.4
Vitamins	100% MS	100% MS	100% MS	50% MS
<i>Myo</i> -inositol (mg L <sup>-1</sup> )	–	100	100	100
Sucrose (g L)	20	20	30	20
BA (mg L <sup>-1</sup> )	0.5	0.5	1.0	–
IBA (mg L <sup>-1</sup> )	0.1	0.05	0.05	0.05
Agar (g L <sup>-1</sup> )	–	5	6	6
Other ingredients	8-Hydroxyquinoline 140 mg L <sup>-1</sup>  Citric acid 210 mg L <sup>-1</sup>	Glucose 5 g L <sup>-1</sup> Fructose 5 g L <sup>-1</sup> PVP 360 100 mg L <sup>-1</sup> PPM™ 0.5 mL L <sup>-1</sup>	–	Arginine 200 mg L <sup>-1</sup>
pH	5.6	5.6	5.6	5.6

MS macronutrients: NH<sub>4</sub>NO<sub>3</sub> 1,650 mg L<sup>-1</sup>, KNO<sub>3</sub> 1,900 mg L<sup>-1</sup>, CaCl<sub>2</sub>·2H<sub>2</sub>O 440 mg L<sup>-1</sup>, MgSO<sub>4</sub>·7H<sub>2</sub>O mg L<sup>-1</sup>, KH<sub>2</sub>PO<sub>4</sub> 170 mg L<sup>-1</sup>; MS micronutrients: NaFeEDTA 36.7 mg L<sup>-1</sup>, KI 0.83 mg L<sup>-1</sup>, H<sub>3</sub>BO<sub>3</sub> 6.3 mg L<sup>-1</sup>, MnSO<sub>4</sub>·4H<sub>2</sub>O 22.3 mg L<sup>-1</sup>, ZnSO<sub>4</sub>·7H<sub>2</sub>O 8.6 mg L<sup>-1</sup>, Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O 0.25 mg L<sup>-1</sup>, CuSO<sub>4</sub>·5H<sub>2</sub>O 0.025 mg L<sup>-1</sup>, CoCl<sub>2</sub>·6H<sub>2</sub>O 0.025 mg L<sup>-1</sup>; MS vitamins: glycine 2.0 mg L<sup>-1</sup>, thiamine HCl 0.1 mg L<sup>-1</sup>, pyridoxine HCl 0.5 mg L<sup>-1</sup>, nicotinic acid 0.5 mg L<sup>-1</sup>

induction on regenerated shoots. Media formulations are given in Table 1.

- 15–75 mL capacity test tubes with caps (16×75, 25×150 mm) or 25–50 mL conical Erlenmayer's flasks, aluminum packaging foil (0.04 mm thick) for culture initiation and first subcultures, 300–500 mL glass jars closed with autoclavable, transparent lids (or adequate culture vessels) for shoot proliferation and rooting, plastic, thermostable (0.1–3 L) beakers.
- Culture room with air conditioning. The possibility of temperature (22–28°C), light intensity (30–50 μmol m<sup>-2</sup> s<sup>-1</sup>)

PPFD), and photoperiod (16/8; 4/2 h day/night) regulation is advantageous. The white growth shelves with white fluorescent lamps, preferentially with bottom-cooling system.

#### **2.4. Acclimation of Regenerated Plants to Ex Vitro Conditions**

1. Greenhouse or plastic tunnel with facilities or as a last resort well separated culture room.
2. Mist chambers (9% RH), multi-cell plug trays with transparent plastic covers/lids. Cell dimensions about 2×3 cm (width, depth), transparent plastic foil.
3. Peat and perlite mixture (2:1 v:v).
4. SCOTTS Peters Plant Starter (10+52+10), calcium carbonate to regulate pH of peat mixture (5.6), fungicides (Previcur 607 SL, Rovral Flo 255 SC).

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### **3. Methods**

The genera *Aronia* and *Malus* belong to the subfamily *Pomoideae*. Thus, the modified micropropagation technique for apple rootstocks could be applied in chokeberry in vitro cultures. The protocol of *Aronia* propagation involves typical three or four stages. The first two stages are the same: the preparation of source material and establishment of axenic cultures (stage I), and multiplication of shoots (stage II) on solid medium with application of double-phase one in the last passage before shoot rooting. The double diluted MS or full strength MS medium with elevated 50% Ca<sup>2+</sup> and Mg<sup>2+</sup> content, supplemented with 0.5–1.0 mg L<sup>-1</sup> 6-benzyladenine (BA), and 0.05 mg L<sup>-1</sup> indole-3-butyric acid (IBA) are used in the first and second stage, respectively. Regenerated shoots treated with IBA (3.0 g L<sup>-1</sup>) could be rooted ex vitro in peat and perlite substrate (stage III+IV, 3-stage method) or rooted in vitro on double diluted MS with 0.05 mg L<sup>-1</sup> IBA (stage III) and then acclimatized in vivo (stage IV, 4-stage method). Obtained microplants are transferred to the tunnel or greenhouse. Weaned, fast growing chokeberry plants could even be planted in the nursery field in the late spring the same year.

#### **3.1. Preparation and Sterilization of Culture Media**

1. All media used in the aronia micropropagation are the modifications of MS medium (10). They differ in concentration of macronutrients and auxins, cytokinins, and other substances (Table 1).
2. Prepare medium from stock solutions (or ready-made powdered one) and reverse-osmosis (or distilled) water. Add medium-specific substances and fix the final volume with water. Adjust the acidity (pH=5.6) with 1 M HCl and 1 M NaOH before addition of agar, as required.

3. When agar is dissolved after medium heating, dispense it into suitable containers, e.g., 2.5–10 mL aliquots into 15–75 mL test tubes for culture initiation, 50 mL aliquots into 300 mL jars for shoot multiplication and rooting, or 100 mL (without agar) into 250–300 mL screw capped jars to prepare double-phase medium.
4. Sterilize media by autoclaving at 121°C for 15, 20 or 25 min.
5. Store the media in the refrigerator up to 2 months if not used.

### **3.2. Plant Source Material and Surface Sterilization**

1. Take rooted parental shoots (young plants) from nursery and plant them in plastic pots (ca 2 L volume) filled with universal potting medium containing compost. Place plants in the shade at room temperature. When plants are not available, collect shoots from parental plants at late winter / early spring. Clean shoots in tap water and spray with fungicides (Bayleton 5 WP, Topsin M). Immerse lower parts of shoots in “forcing medium” (Table 1). Place shoots in the shade at room temperature to obtain etiolated sprouts.
2. Tear off 5–10 cm sprouts; remove leaf blades (without petioles).
3. Wash shoots for 10 min under cold running tap water with detergent and surface sterilize them by immersion in 70% (v:v) ethanol for 1 min, followed by shaking them in a clean jar containing commercial bleach diluted with water (1:5 v:v) for 20 min. Use laboratory shaker or ultrasonic washer if available.
4. Wash shoots three times with sterile reverse-osmosis water for 5 min.

### **3.3. Culture and Maintenance of Explants**

1. After sterilization, make nodal explants (about 10 mm long) using a sterile scalpel and place them individually on the initiation medium (Table 1) in small test tubes (Fig. 1a, b).
2. Prepare 20 explants per genotype.
3. Excise new axillary sprouts which emerged in vitro. Make new explants (nodal sections with or without shoot tip, 6–10 mm long) and place again on initiation medium. Repeat it every 2–4 weeks to maintain active growth and select healthy cultures (see Note 2, Fig. 1c).

### **3.4. Multiplication of Shoots**

1. Subculture explants (1 cm long) on proliferation (c) medium (Table 1) every 5–6 weeks from third-fourth passage. Use 300 mL capacity glass jars closed with transparent lids or adequate culture vessels. Place 12–16 nodal explants about 1 cm long in each vessel (see Notes 3 and 4).
2. In the last passage (before rooting) make double-phase medium (d, e media) to stimulate elongation of shoots.



Fig. 1. Propagation of chokeberry through in vitro cultures. (a, b) Initiation of in vitro culture, (c, d) maintained culture of aronia, (e) growth of in vitro cultures on double-phase “2” and solid “1” media, (f, g) rooted shoots in vitro, (h, i) regenerated plant of chokeberry. (e–g) Copyright by Wrocław University of Environmental and Life Sciences (7).

For this purpose pour 5–10 mL liquid (e) medium onto solid one (d) (Table 1), (see Note 5).

3. Grow the cultures at  $26 \pm 1^\circ\text{C}$  and 16 h/8 h day/night photoperiod under cool-white light at about 20 (initiation)— $30 \mu\text{mol m}^{-2} \text{s}^{-1}$  PPFD.

### 3.5. Acclimation of Regenerated Shoots to Ex Vitro Conditions

1. Place healthy shoots (1.5 cm long) onto rooting medium (see Notes 6 and 7; Table 1). After 2 weeks, root primordial and first short roots are visible. Clean roots carefully by removing agar with lukewarm tap water. Transfer rooted shoots into peat and perlite mixture (2:1 v:v; pH=5.8) watered with fertilizer “SCOTTS Peters Plant Starter” solution (0.8 gL<sup>-1</sup>) and fungicide Previcur 607 SL (0.15%). Spray shoots with fungicide Rovral Flo 255 SC (0.1%) solution. Use multi-cell plug trays with transparent plastic covers/lids. Grow plants at high air relative humidity (95% RH) in 16 h/8 h day/night photoperiod under sodium light at 60–100 μmol m<sup>-2</sup> s<sup>-1</sup> PPFD at 20–24°C. Protect plants from direct intense solar radiation. After 2–3 weeks plastic lids should be gradually lifted up and finally removed. Transfer weaned, fast growing plants to the nursery tunnels or field.
2. The rooting in vitro stage could be omitted (see Note 8), by using 2 cm long shoots. Dip shoot base in water-ethanol (1:1 v:v) rooting solution of IBA (3.0 gL<sup>-1</sup>). Place them directly in pots filled with peat and perlite mixture. Treat them with similar, aforementioned method.

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## 4. Notes

1. Use cultivar supplied either by the breeder/owner or a reliable nurseryman. However, it is recommended to establish own collections of healthy parental plants.
2. Chokeberry clones readily adjust to in vitro conditions and change into rapidly growing cultures in second–third initiation passage (Fig. 1c, d). However, shoots harbor endogenous bacteria which contaminate medium even in fourth passage. Therefore addition of 0.5 mL L<sup>-1</sup> PPM (plant preservative mixture) in the culture medium is recommended.
3. The propagation ratio is high. From one 2-node explant, more than 9 shoots (which could be divided into more than 20 new explants) or more than 6 shoots suitable for rooting can be expected (Fig. 1d). Thus more than 100 shoots from one jar may be obtained (Fig. 1e).
4. The chokeberry cultures at the proliferation stage can be easily stored in refrigerator for more than 1 year.
5. The double-phase medium strongly stimulates shoot elongation, but does not significantly influence shoot proliferation (Fig. 1e).
6. Although it is more complicated and expensive, the two-step procedure (rooting shoots in vitro and acclimation them in vivo) can be applied. The rooting passage lasts 2–3 weeks.

More than 90% shoots root *in vitro* (Fig. 1f, g). A similar procedure may be applied during *ex vitro* rooting of the shoots. The survival rate reaches near 100% (Fig. 1h, i).

7. Shoots with tips root slightly better than nodal segments both *in vivo* and *in vitro*.
8. Rooting shoots *ex vitro* may be used. The IBA treatment of shoots does not improve efficiency of *in vivo* rooting (more than 70%) but promotes the further growth of obtained plants.

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# **Part II**

## **Protocols for Micropropagation of Ornamentals and Cut Flowers**

## Micropropagation of *Lavandula* spp.

Sandra Gonçalves and Anabela Romano

### Abstract

*Lavandula* species are some of the most popular ornamental and medicinal plants with great economic values. These species are vegetative propagated by stem cuttings. However, the poor rooting ability and vulnerability of plantlets to contamination are major limiting factors for propagation. In vitro culture methods are suitable to overcome these limitations. This chapter describes protocols for in vitro propagation of *Lavandula viridis* L'Hér and *Lavandula vera* DC. Nodal shoot proliferation of *L. viridis* and plant regeneration from leaf-derived callus of *L. vera* by an “open culture system” are highlighted.

**Key words:** Aromatic plants, In vitro culture, *L. vera*, *L. viridis*, Medicinal species

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### 1. Introduction

*Lavandula* species are members of the Lamiaceae family. They are native to the Mediterranean region, southern tropical Africa and southeast regions of India (1). The genus includes annuals, herbaceous plants, and small shrubs, having aromatic foliage and flowers. Several *Lavandula* species are cultivated worldwide as ornamental and medicinal plants (2). The essential oils of these species are highly valuable for cosmetic, food, and pharmaceutical industries. The insecticidal (3) and phytotoxic properties (4) of some species are of great importance to the agro-chemical industries.

Seed propagation of *Lavandula* species is usually slow, with low and sporadic germination rates. Moreover, seedlings are variable in growth habit, color, and oil composition; desirable traits may not always be retained. By stem cuttings, vegetative propagation is faced with major hurdles such as poor rooting ability and vulnerability to contamination (5–7); restrain commercial use of conventional propagation techniques for large-scale plant production. In vitro propagation methods are most suitable to overcome these limitations. By micropropagation, rapid propagation is carried

out successfully for large-scale production of uniform plants of valuable genotypes (8). Moreover, plant regeneration through organogenesis and somatic embryogenesis facilitate clonal propagation and genetic transformation of genotypes by *Agrobacterium* and particle bombardment-mediated gene delivery. These technological developments would improve essential oil composition and productivity of *Lavandula* species (9); certainly increase economic potential in pharmaceutical and floriculture industries.

Micropropagation protocols using hypocotyl sections, shoot tips, or nodal explants have been reported in several *Lavandula* species: *L. latifolia* (6, 10), *L. stoechas* (7), *L. dentata* (8, 11), *L. viridis* (12), and *L. vera* (*Lavandula vera* DC=*Lavandula officinalis* Chaix=*Lavandula angustifolia* Mill.) (13, 14). Plant regeneration from leaf-derived callus was reported in *L. vera* (2, 15–17) and in lavandin (9), an hybrid between *L. vera* and *L. latifolia*.

In this chapter, two distinct protocols are described: (1) in vitro propagation of *L. viridis* through nodal shoot proliferation (12) and (2) regeneration of *L. vera* plants from leaf-derived callus (16). *L. viridis*, commonly known as green or white lavender, is a xerophytic aromatic shrub, endemic to the south-western Iberian Peninsula, growing in dry conditions and poor degraded soils. The essential oils and volatiles obtained from *L. viridis* field-grown and in vitro plants consist of mainly monoterpenes with the major components 1,8-cineole and camphor (18, 19). Common lavender (*Lavandula vera* DC=*Lavandula officinalis* Chaix=*Lavandula angustifolia* Mill.) is important both as ornamental and essential oil producing plant, and native to the western Mediterranean region. Lavender essential oil has high amount of linalool and linalyl acetate, moderate levels of lavandulyl acetate, terpinen-4-ol, and lavandulol (20, 21). The amount of 1,8-cineole and camphor often varies from very low to moderate (22). In both *Lavandula* species, the oil quality is genotypic dependent; therefore in vitro propagation of selected genotypes is a requisite for their commercial production.

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## 2. Materials

### 2.1. In Vitro Propagation of *L. viridis* L'Hér from a Single Node

#### 2.1.1. Surface Sterilization of Plant Material

1. Sterile distilled water.
2. Commercial bleach solution 20%, v/v in sterile distilled water (about 2%, w/v, active chlorine).
3. Ethanol 70% (v/v).
4. Tissue culture facilities: instruments (scalpel and forceps), Petri dishes, dry glass bead sterilizer, autoclave, laminar flow bench, culture room.

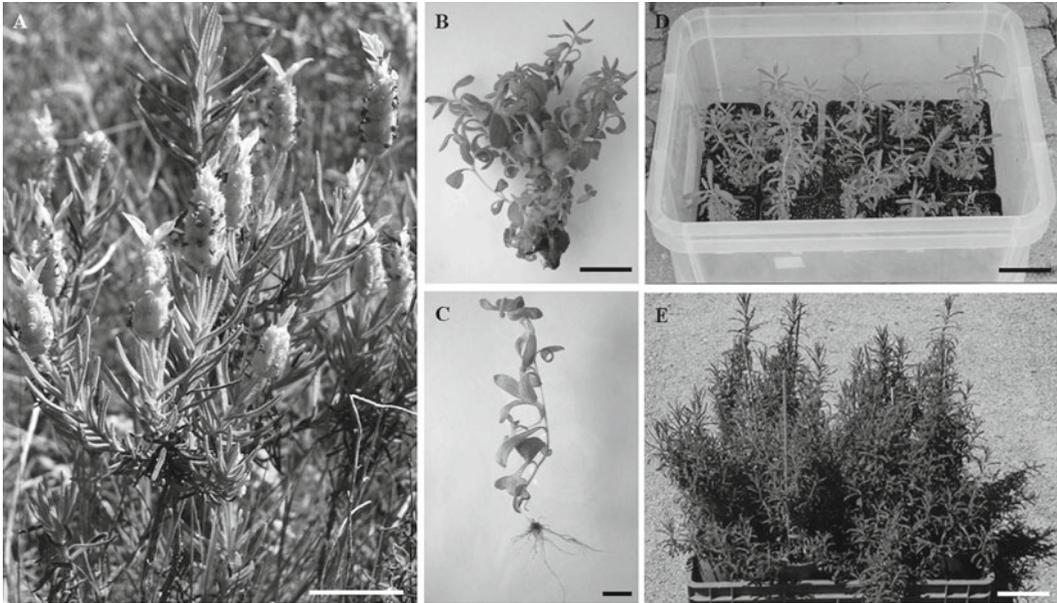


Fig. 1. Micropropagation of *L. viridis*. (a) Field specimen (bar, 5 cm), (b) shoots at the end of the multiplication phase in  $\frac{1}{2}$ MS medium with  $0.67 \mu\text{M}$  BA (bar, 1 cm), (c) rooted shoot in GD medium with  $10.74 \mu\text{M}$  NAA (bar, 1 cm), (d) plants at the end of the acclimatization stage (bar, 5 cm), (e) plantlets after 5 months ex vitro (bar, 10 cm).

5. 250 mL screw capped bottles (autoclaved).
6. Magnetic stirrer, magnetic bar, 500 mL beaker (autoclaved).
7. Autoclaved Whatman No. 1 filter paper.
8. Selected field-grown plant as explant source (Fig. 1a).

### 2.1.2. Culture Media

1. Murashige and Skoog medium (MS; (23)) for in vitro establishment,  $\frac{1}{2}$ MS medium for shoot multiplication, and Gresshoff and Doy medium (GD; (24)) for root induction (Table 1).
2. Growth regulators:  $\text{N}_6$ -benzyladenine (BA) for shoot culture establishment and multiplication, and naphthalene acetic acid (NAA) for rooting.
3. Sucrose (Panreac, Spain, Barcelona) and agar (Iberagar, Coima, Portugal).
4. Test tubes ( $25 \times 160$  mm), transparent polyethylene caps.

### 2.1.3. Acclimatization of Micropropagated Plants to Ex Vitro Conditions

1. Tap water.
2. Quarter-strength MS solution.
3. Plastic pots (6 cm diameter, 100 mL capacity).
4. Potting medium, consisting of a mixture of peat and vermiculite (1:1, v/v).
5. Transparent polyethylene boxes.

**Table 1**  
**Composition of MS (23) and GD (24) media**

Component	Concentration (mg L <sup>-1</sup> )	
	MS	GD
NaH <sub>2</sub> PO <sub>4</sub> ·H <sub>2</sub> O	–	90
Na <sub>2</sub> HPO <sub>4</sub>	–	30
KNO <sub>3</sub>	1,900	1,000
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	–	200
MgSO <sub>4</sub> ·7H <sub>2</sub> O	370	250
CaCl <sub>2</sub> ·2H <sub>2</sub> O	440	150
NH <sub>4</sub> NO <sub>3</sub>	1,650	–
KH <sub>2</sub> PO <sub>4</sub>	–	–
KCl	–	300
MnSO <sub>4</sub> ·H <sub>2</sub> O	–	10
MnSO <sub>4</sub> ·4H <sub>2</sub> O	22.30	–
H <sub>3</sub> BO <sub>3</sub>	6.20	3
ZnSO <sub>4</sub> ·7H <sub>2</sub> O	8.60	3
Na <sub>2</sub> MoO <sub>4</sub> ·2H <sub>2</sub> O	0.25	0.25
KI	0.83	0.75
CoCl <sub>2</sub> ·6H <sub>2</sub> O	0.025	0.25
CuSO <sub>4</sub> ·5H <sub>2</sub> O	0.025	–
Na <sub>2</sub> EDTA·2H <sub>2</sub> O	37.25	37.25
FeSO <sub>4</sub> ·7H <sub>2</sub> O	27.85	27.85
Myo-inositol	100	10
Nicotinic acid	0.5	0.1
Pyridoxine HCl	0.5	0.1
Thiamine HCl	0.1	1
Glycine	2	0.4

## 2.2. In Vitro

### **Propagation of *L. vera* DC from Leaf-Derived Callus Using the “Open Culture System” (16)**

#### 2.2.1. Surface Disinfection of Plant Material

1. Sodium hypochlorite solution, about 2% active chlorine.
2. Plants growing in the greenhouse, 25–30 °C, natural day-length, about 500–700 μmol/m<sup>2</sup>/s, which are the source of explants.

### 2.2.2. Culture Media

1. MS media for callus induction, initiation and development of multiple shoots, and  $\frac{1}{2}$ MS for rooting (Table 1).
2. Growth regulators: 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin (KIN) for callus induction, *N*-(chloro-4-pyridyl)-*N'*-phenylurea (CPPU) for induction and development of multiple shoots, and indoleacetic acid (IAA) for rooting.
3. Gelling gum.
4. Test tubes (18 × 180 mm), culture flasks 200 mL capacity, aluminum foil and membrane filter (0.25  $\mu$ m pore size, Iwaki, Japan).

### 2.2.3. Acclimatization of Regenerated Plants to Ex Vitro Conditions

1. Hyponex<sup>®</sup> solution ( $\times 1/2,000$ ).
2. Top-sealed plastic bottles.
3. Potting medium consisting of vermiculite.

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## 3. Methods

### 3.1. In Vitro Propagation of *L. viridis* from Single-Node Explants

Micropropagation of *L. viridis* from nodal shoot proliferation involves four steps: (1) selection of plant material and establishment of axenic cultures from single-node explants; (2) multiplication of shoots obtained at the end of the establishment stage (4 weeks) on  $\frac{1}{2}$ MS medium supplemented with 0.67  $\mu$ M BA; (3) root induction in well developed shoots on GD medium containing NAA; and (4) acclimatization of micropropagated plants to ex vitro conditions with a gradual reduction of the humidity.

#### 3.1.1. Preparation and Sterilization of Culture Media

1. Prepare media for culture establishment from single-node explant, shoot culture proliferation, maintenance, and rooting (Table 1). Media for culture establishment and shoot multiplication contain 0.44 and 0.67  $\mu$ M BA, respectively. The rooting medium contains 10.74  $\mu$ M NAA. Add appropriate volumes of stock solutions of all the ingredients, including plant growth regulators, to give the required final concentrations (see Note 1).
2. Add 2% (w/v) sucrose to establishment and multiplication media, and 3% (w/v) to rooting medium. Add 0.7% (w/v) agar to all media.
3. Adjust pH of the media to 5.8 using 1 M HCl or 1 M NaOH.
4. Heat agar in microwave oven until the solution is clear.
5. Distribute media into test tubes (25 × 160 mm, 10 mL/tube).
6. Sterilize media by autoclaving at 121 °C for 20 min (1 bar).
7. Store autoclaved media at the room temperature for a maximum of 1 month in the darkness.

### 3.1.2. Plant Material and Surface Sterilization

1. Excise single-node explants (10–12 mm long) from new actively growing shoots of a mature field-grown plant. Spring (March–April) is the best season for explant collection for culture initiation.
2. Disinfect explants with 70% ethanol for 2 min, followed by immersion in commercial bleach (20%, v/v) for 15 min in a sterile beaker on a magnetic stirrer. Autoclave magnetic bars, screw capped bottles and beakers before use, and dry in a drying oven (see Note 2).
3. Wash explants several times in sterile distilled water and dry them on sterile (autoclaved) filter paper.

### 3.1.3. Cultures Establishment and Multiplication

1. Culture explants individually in test tubes, containing 10 mL of establishment medium, and close with transparent polyethylene caps.
2. Incubate at  $25 \pm 2$  °C with a 16 h photoperiod, light intensity,  $60 \mu\text{mol}/\text{m}^2/\text{s}$ , during 4 weeks.
3. For multiplication, separate shoots obtained at the end of the establishment stage (see Note 3) and divide into single-node explants. Inoculate explants into  $\frac{1}{2}\text{MS}$  medium containing  $0.67 \mu\text{M}$  BA.
4. Grow them for 4 weeks under the growing conditions described in the previous step 2 (see Note 4).

### 3.1.4. Rooting of Micropropagated Shoots

1. At the end of multiplication stage (Fig. 1b) excise well developed shoots (30–40 mm high); transfer to GD medium amended with 3% (w/v) sucrose; and add  $10.74 \mu\text{M}$  NAA in the root induction medium (see Note 5).
2. Maintain cultures for 4 weeks under the similar growing conditions as described in the previous step (see Note 6).

### 3.1.5. Acclimatization of Micropropagated Plants to Ex Vitro Conditions

1. Remove rooted shoots with minimum three well-developed roots from the culture medium. Wash roots with tap water in order to remove agar sticking to roots without any damage and also to prevent pathogenic contaminations (Fig. 1c).
2. Transfer each individual plant to 6 cm diameter plastic pots, containing a mixture of moist peat and vermiculite (1:1, v/v).
3. Cover plants with transparent polyethylene boxes and maintain in growth room under controlled conditions, 16 h photoperiod,  $60 \mu\text{mol}/\text{m}^2/\text{s}$ ,  $25 \pm 2$  °C, and 90% relative humidity.
4. After 2 weeks, remove plastic covers to gradually expose plants to reduced relative humidity and facilitate the acclimatization of plants to ex vitro conditions. After 2 more weeks, 80% plants are adequately acclimatized (Fig. 1d) and can be transferred to the glasshouse (Fig. 1e) (see Note 7).
5. While acclimatization, water plants at 3-day interval with  $\frac{1}{4}\text{MS}$  solution.

**3.2. Plant Regeneration of *L. vera* from Leaf-Derived Callus Using The “Open Culture System” (16)**

This protocol describes an efficient procedure for plant regeneration from leaf-derived callus of *L. vera* using multiple shoots and an “open culture system” (16). In this system the culture flasks were sealed with aluminum foil having a small hole covered with a membrane filter allowing the exchange of the gas and vapor. The protocol involves: (1) establishment of axenic cultures of leaf segments and callus production on medium containing 1  $\mu\text{M}$  2,4-D and 1  $\mu\text{M}$  KIN; (2) multiple shoot formation from callus cultivated in medium supplemented with CPPU; (3) the development of multiple shoots in the same medium but using an “open culture system.” In this system culture flasks are sealed with aluminum foil; make small holes (7×7 mm) covered with a membrane filter (0.25  $\mu\text{m}$  pore size, Iwaki, Japan); (4) root induction in IAA supplemented medium, using the “open culture system”; and (5) acclimatization of regenerated plants to ex vitro conditions with a gradual reduction of humidity. The great advantage of this protocol is that the production of multiple shoots using the “open culture system” increases the plant regeneration rate about seven times as compared with the normal shoot culture system.

**3.2.1. Preparation and Sterilization of Culture Media**

1. Prepare MS culture media for callus induction, induction and development of multiple shoots and rooting (Table 1). Callus induction medium: 1  $\mu\text{M}$  2,4-D and 1  $\mu\text{M}$  KIN; induction and development of multiple shoots media: 0.4  $\mu\text{M}$  CPPU (see Note 8); rooting medium: 1  $\mu\text{M}$  IAA.
2. Add 3% sucrose and 0.3% gellan gum to the media.
3. Distribute media into suitable containers, e.g., test tubes (18×180 mm) for callus induction, or 200 mL culture flasks for induction and development of multiple shoots and rooting.

**3.2.2. Plant Material and Surface Disinfection**

1. Excise lateral branches with fully developed leaves, about 5 cm long.
2. Immerse plant material in 70% ethanol for 1 min, followed by soaking in sodium hypochloride solution for 20 min. Wash plant material three times in sterilized distilled water.
3. Detach leaves and cut into small segments (3×5 mm long).

**3.2.3. Callus Induction, Multiple Shoots Induction, and Development**

1. Inoculate leaf segments in test tubes containing 10 mL callus induction medium.
2. Incubate cultures in the darkness at 25 °C for callus induction.
3. After 40 days, transfer calli (about 7×7 mm in size) to MS medium containing 0.4  $\mu\text{M}$  CPPU for induction of multiple shoots.
4. Incubate 10 weeks under continuous cool-white fluorescent light, 70  $\mu\text{mol}/\text{m}^2/\text{s}$ , 25 ± 1 °C.

5. Transfer adventitious shoots, obtained by organogenesis, to 200 mL culture flasks containing 50 mL same culture medium, under the conditions described above.
6. After 2 weeks, divide multiple shoot clusters (about 3–3.5 cm in diameter) (see Note 9) into 7–20 shoot clusters with 2–3 shoots (about 7×7 mm in size) and subculture on fresh medium every 2 weeks till shoots grow up to 1 cm in height.
7. During subculture use an “open culture system” in order to exchange gas and vapor generated in the flasks. For that, seal the flask with aluminum foil having a small hole (7×7 mm) covered with a membrane filter (0.25 µm pore size, Iwaki, Japan) (see Note 10).

#### *3.2.4. Root Induction of Regenerated Shoots*

1. Select shoot clusters with shoots 1 cm or longer and transfer to ½MS containing 1 µM IAA (see Note 11).
2. Use the “open culture system” (see Note 12) and incubate under cool-white fluorescent light, 70 µmol/m<sup>2</sup>/s, 25 °C.
3. After 4 weeks, about 70% shoots develop roots.

#### *3.2.5. Acclimatization of Micropropagated Plants to Ex Vitro Conditions*

1. Transplant rooted shoots to top-sealed plastic bottles, containing vermiculite moistened with Hyponex® solution.
2. Decrease humidity in bottles within 2 weeks by opening the seals.
3. Transfer acclimatized plants to soil in pots and maintain in the greenhouse at 25–30 °C with natural day length and light intensity 500–700 µmol/m<sup>2</sup>/s.

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## 4. Notes

1. Prepare stock solutions (e.g., 50 or 100×) of all ingredients and store at 4 °C. Stock solutions can also be stored at –20 °C for longer time. Use only thick walled glass bottles (e.g., the Duran type) while microwaving the frozen culture media. Prepare stock solutions of macro and micro salts, iron, and additives; prepare stock solutions of BA and NAA at a concentration of 2.22 and 2.69 µM, respectively. Dissolve compounds in small volumes of 1.0 M NaOH and adjust volume with water. Store stock solutions of plant growth regulators maximum up to 1 month in the darkness at 4 °C.
2. The initiation of cultures is difficult due to microbial contamination. The use of described method for surface sterilization can obtain around 50% surface-disinfected explants.

3. Axillary shoot development should be observed in 95% uncontaminated explants after 12–15 days. Callus formation at the base of the explant and organogenesis can occur.
4. At the end of the multiplication stage, on an average 12 shoots per initial nodal explant are obtained; longest shoots are 4–4.5 cm in height.
5. By increase sucrose concentration from 2 to 3% (w/v) in the rooting medium, rooting frequency rate increases from 40 to 84%. NAA is the preferred auxin for inducing roots on micropropagated shoots of *L. viridis*.
6. Four weeks after rooting induction, over 80% shoots developed roots.
7. In principle, all surviving plants must show a high degree of homogeneity. According to Nogueira and Romano (18), GC-MS analysis showed no variation in composition of essential oils both in field-grown mother plant and in vitro shoot cultures or acclimatized plants of the same clone.
8. CPPU (a purine-type cytokine) performs better than BA in inducing abundant normal shoots (15).
9. Division of shoot clusters is recommended, as shoots develop more vigorously.
10. Multiple shoots growing in a “closed-system” became vitrified and had poor growth. This problem can be overcome by using the “open-system” in which shoots did not vitrify and grow vigorously.
11. IAA must be filter-sterilized and add to the autoclaved medium just before solidification.
12. “Open culture system” increases rooting frequency rate and number of roots per shoot; also improves acclimatization rate of regenerated plants. About 95% plants survive during acclimatization.

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## In Vitro Propagation of *Acacia mangium* and *A. mangium* × *A. auriculiformis*

Olivier Monteuis, Antoine Galiana, and Doreen Goh

### Abstract

*Acacia mangium* and *A. mangium* × *A. auriculiformis* hybrids have gained an increasing interest in reforestation programs under the humid tropical conditions, mainly for pulpwood production. This is due to their impressive growth on acid and degraded soils, as well as their capability to restore soil fertility thanks to their natural nitrogen-fixing ability. It is crucial to develop efficient methods for improving the genetic quality and the mass production of the planting stocks of these species. In this regard, in vitro micropropagation is well suited to overcome the limitations of more conventional techniques for mass propagating vegetatively selected juvenile, mature, or even transgenic genotypes. Micropropagation of *A. mangium* either from seeds or from explants collected from outdoors is initiated on Murashige and Skoog (MS) basal medium supplemented with 4.4 μM BA. Microshoot cultures produced by axillary budding are further developed and maintained by regular subcultures every 60 days onto fresh MS culture medium added with 2.2 μM BA + 0.1 μM NAA. This procedure enhances the organogenic capacity for shoot multiplication by axillary budding, with average multiplication rates of 3–5 every 2 months, as well as for adventitious rooting. The rooting is initiated on Schenk and Hildebrandt culture medium containing 4 μM IAA. The maintenance of shoot cultures in total darkness for 3 weeks increases the rooting rates reaching more than 70%. The hybrid *A. mangium* × *A. auriculiformis* genotypes are subcultured at 2-month intervals with an average multiplication rate of 3 and rooting rates of 95–100% on a half-strength MS basal medium containing 1.1 μM NAA. The rooted microshoots are transferred to ex vitro controlled conditions for acclimatization and further growth, prior to transfer to the field, or use as stock plants for cost-effective and true-to-type mass production by rooted cuttings.

**Key words:** *Acacia* hybrids, Adventitious rooting, Axillary budding, Cloning, Legume tree, Micropropagation

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## 1. Introduction

*Acacia* genus belongs to the Leguminosae family that encompasses some 1,300 tree and shrub species, mainly native to Australia and to the tropical and warm-temperate regions of both hemispheres including Africa, southern Asia, and the Americas. As

legumes, *Acacia* species have the natural ability to fix atmospheric nitrogen and hence can thrive in low fertility soils (1, 2). They can be used for combating desertification or for restoring degraded lands (*A. tortilis*, *A. nilotica*), and also for producing gum (*A. senegal*), tannin (*A. mearnsii*), fodder, and also wood for different end-uses (3–6). Both *A. mangium* and *A. auriculiformis* originate from Papua New Guinea (PNG), eastern provinces of Indonesia and northeast Queensland in Australia (7). These two arborescent species can hybridize naturally to give rise to the interspecific hybrid *A. mangium* × *A. auriculiformis*. This hybrid is more site-adaptable, produces higher yield of denser wood and cuttings with higher capacity for adventitious rooting than *A. mangium*. For all these reasons and also due to the severe drawbacks associated with seed propagation of interspecific hybrids, the mass clonal propagation of *A. mangium* × *A. auriculiformis* superior genotypes is of strategic importance. Since their introduction as exotics in many tropical countries, especially in South-East Asia, *A. mangium*, *A. auriculiformis* and the hybrids *A. mangium* × *A. auriculiformis* have gained increasing popularity for reforestation programs (7–9). This striking expansion of *Acacia spp.* plantations within a few years, mainly for pulp-wood production, is due to their impressive growth performance under humid tropical conditions, especially on acid and degraded soils, as well as to their capability to restore soil fertility thanks to their natural nitrogen-fixing ability (10–14). There is therefore a crucial need to develop efficient methods for mass producing superior-quality or transgenic planting stocks of these species (6, 15). In this respect, the in vitro techniques, specifically micropropagation (16–18), are worth considering for overcoming the limitations of more conventional nursery methods used for vegetatively mass propagating superior genotypes, especially mature ones (19–21).

The aim of this chapter is to provide protocols for sustainable micropropagation of the highly economically important *A. mangium* and the interspecific hybrid, *A. mangium* × *A. auriculiformis*. Stepwise information on the successive phases of primary culture initiation, establishment of organogenic cultures, rooting, and ex vitro acclimatization suitable for juvenile and mature selected genotypes is provided. Such information can also be useful for genetic engineering (6, 22–24).

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## 2. Materials

### 2.1. Sterilization of Outdoor-Grown Selected Plant Material

1. Select mother plants growing naturally outdoors, or hedged stock plants in the nursery, or seeds.

2. Shears, secateurs (or averruncator or a ladder or a climber for not easily accessible shoots), ice box, plastic bags, moistened newspaper, permanent markers or pencils, notebooks.
3. Tap water.
4. Ethanol 70% and 95% (v:v).
5. Wetting agent such as dishwashing liquid soap or Tween 20.
6. Commercial bleach solution (NaClO) diluted 1:10 (v:v) with tap water, mercuric chloride (HgCl<sub>2</sub> 1 and 2 mg/L).
7. Autoclaved pure or ultrapure water (distilled, reverse-osmosis water) in 250 mL screw capped bottles.
8. Autoclaved absorbing paper (filter paper, newspapers).
9. Magnetic stirrer, magnetic bar.
10. Usual tissue culture facilities, equipment and instruments—laminar flow hood, bunsen or ethanol burner, scalpel, forceps, glass culture tubes (21 × 150 mm covered with polypropylene caps).
11. Tea strainer.
12. Binocular microscope and cold light source for shoot apex excision.
13. Cellulosic or autoclave-resistant and chemically neutral 20 × 30 mm plugs (Sorbarod).
14. Specific dissecting tools (mounted razor blade splinter).

### **2.2. In Vitro Culture**

1. Glass culture tubes held in appropriate racks with polypropylene or polycarbonate caps.
2. Glass culture jars and flasks (total capacity of 300–800 mL) with appropriate polypropylene or polycarbonate caps.
3. General medium preparation equipment (pH meter, precision and analytical balances, macro- and equipment micro-salts required for Murashige and Skoog (MS (25)), and Schenk and Hildebrandt (SH (26)) media (see Table 1).
4. Culture rooms with automatic temperature control ( $26 \pm 2^\circ\text{C}$ ) and a 16 h photoperiod ( $50\text{--}60 \mu\text{mol/m}^2/\text{s}$ , “ORSAM L36W/77” fluorescent lamps).

### **2.3. Acclimatization to Ex Vitro Conditions**

1. Bowls and plastic containers, that can be filled with water and aqueous solutions of fungicide for soaking the collected plant material and for transfer from in vitro to ex vitro conditions.
2. Forceps.
3. Rooting substrate (river sand, peat, composted bark) that can be used in mixture.
4. Shade-, lath-, or greenhouse facilities equipped with polyethylene-covered benches or beds, automatic mist or fog system.

**Table 1**  
**Composition (in mg/L) of the basal and rooting media used for micropropagating *Acacia mangium* and the hybrid *A. mangium* × *A. auriculiformis***

	<i>Acacia mangium</i>		<i>A. mangium</i> × <i>A. auriculiformis</i>
	Basal medium	Rooting medium	Basal medium
KNO <sub>3</sub>	1,900	830	950
NH <sub>4</sub> NO <sub>3</sub>	1,650	100	825
KH <sub>2</sub> PO <sub>4</sub>	170		85
CaCl <sub>2</sub> ·2H <sub>2</sub> O	440	70	220
MgSO <sub>4</sub> ·7H <sub>2</sub> O	370	130	185
FeSO <sub>4</sub> ·7H <sub>2</sub> O	27.8	13.7	27.8
Na <sub>2</sub> EDTA	37.3	18.6	37.3
H <sub>3</sub> BO <sub>3</sub>	6.2	3.1	6.2
ZnSO <sub>4</sub> ·7H <sub>2</sub> O	8.6	4.3	8.6
MnSO <sub>4</sub> ·H <sub>2</sub> O	16.9	8.4	16.9
Na <sub>2</sub> MoO <sub>4</sub> ·2H <sub>2</sub> O	0.25	0.12	0.25
KI	0.83	0.41	0.83
CuSO <sub>4</sub> ·5H <sub>2</sub> O	0.025	0.012	0.025
CoCl <sub>2</sub> ·6H <sub>2</sub> O	0.025	0.012	0.025
Myo-inositol	50	50	50
Glycine	2	2	2
Thiamine HCl	1	1	1
Pyridoxine HCl	1	1	1
Nicotinic acid	1	1	1
Casein hydrolysate	200	200	200
BA	0.5 (2.2 µM)		
NAA	0.02 (0.1 µM)	0.7 (4 µM)	0.2 (1.1 µM)
Sucrose	20,000	20,000	20,000
Phytigel	2,500	2,500	2,500

5. Fungicides (thiram, benomyl).

6. Recyclable or disposable containers of various kinds, e.g., plastic bags, root trainers, plastic pots, potting cells, Jiffy pots.

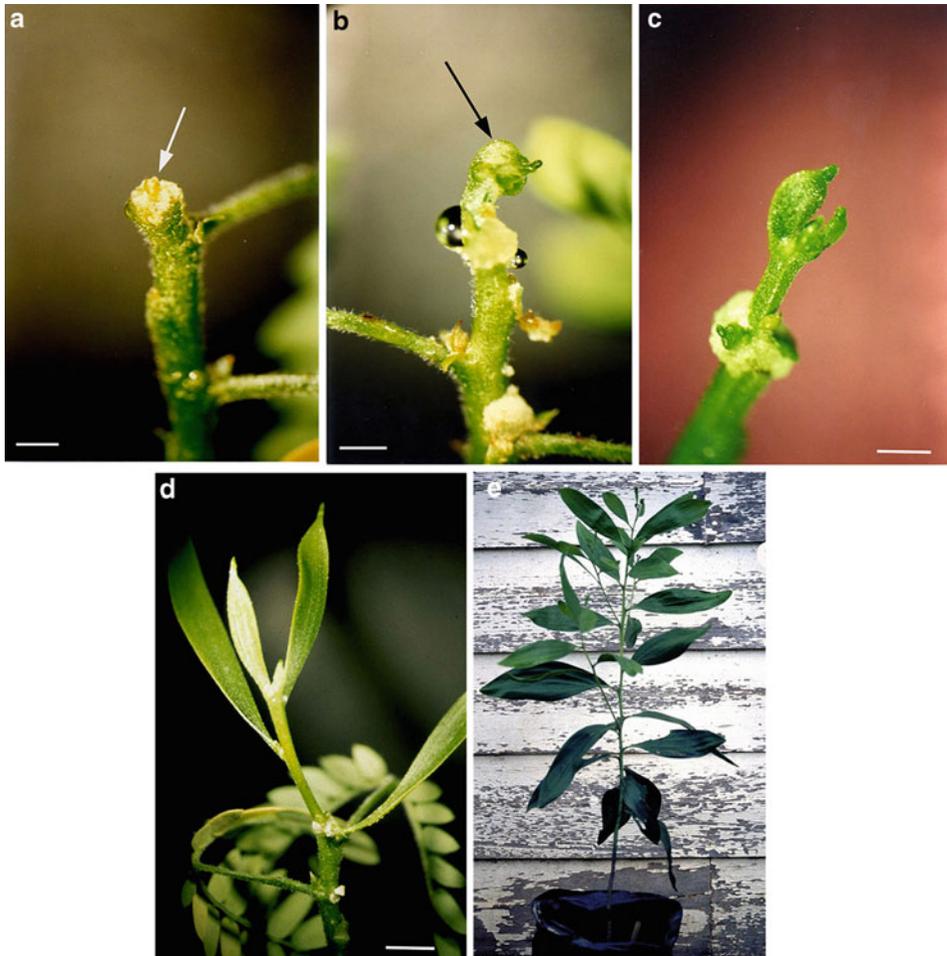


Fig. 1. Shoot apex micrografting of mature *A. mangium*. (a) shoot apex (arrow) newly top grafted in aseptic conditions onto an in vitro-grown seedling rootstock; (b–d) scion elongation; (e) acclimatized micrograft in outdoor conditions. Scale bars correspond to 1 mm for a–c, and to 2 mm for d.

### 3. Protocols

*Acacia* spp. can be micropropagated from in vitro germinated seeds (27, 28), transgenic cells (6, 24), and outdoor explants (18). In the latter case, in vitro introduction can be done directly on the culture medium (29), or by shoot apex micrografting (see ref. (30); Fig. 1).

#### 3.1. Culture Medium Preparation

1. For *A. mangium* culture initiation, prepare MS medium (for details see Table 1) (see Note 1) by using stock solutions (see Note 2). Increasing BA concentration to 4.4  $\mu\text{M}$  during culture initiation stimulates axillary bud development from nodal explants, collected from mature trees. Interspecific hybrid *A. mangium*  $\times$  *A. auriculiformis* cultures require modified MS

medium containing half strength of macronutrients and 1.1  $\mu\text{M}$  NAA (see Table 1 (14, 31)).

2. Add 20 g/L sucrose and stir the medium continuously. Adjust the pH to 5.5–5.7 using 1 M KOH or NaOH.
3. Add 0.25% Phytigel (Sigma, St Louis, w/v) before heating the medium to about 80°C. Dispense 10 mL into glass culture tubes (21 × 150 mm), covered with polypropylene caps (see Note 3).
4. Sterilize culture media by autoclaving at 120°C and 95 kPa for 20 min (see Note 4).
5. Culture explants on solidified culture media under the laminar flow hood to prevent any contamination.
6. For *A. mangium*, the rooting medium SH/3 contains 1/3 strength SH macronutrients, half-strength MS micronutrients, and 4  $\mu\text{M}$  indole-3-acetic acid (IAA) (see Table 1). *A. mangium* × *A. auriculiformis* shoot explants root on their basal medium.

### 3.2. Initiating the Cultures

#### 3.2.1. From Seed

1. Place seeds in tea strainers before soaking 5–10 s in boiling water, followed by immersion for 5 min in 70% ethanol and then for 3 min in 1 mg/L  $\text{HgCl}_2$  aqueous solution (see Note 5).
2. Rinse three times in sterilized ultrapure water under laminar flow hood.
3. Inoculate 2–3 seeds per tube (see Note 6) filled with Phytigel solidified culture medium, or place these seeds on liquid imbibed plugs in the test tubes for rootstock production (see Note 1). Transfer the seed-containing tubes to the culture room under a 16 h photoperiod (50–60  $\mu\text{mol}/\text{m}^2/\text{s}$ , “ORSAM L36W/77” fluorescent lamps) at  $26 \pm 2^\circ\text{C}$ . Maintain one germinated seed per tube; the germinations in excess can be transferred, upon the emergence of the radical, to other individual tubes.

#### 3.2.2. From Outdoor Developed Plants

1. For field or nursery-grown selected acacia trees of any age, collect elongating shoots from the more accessible parts of the tree, preferably close to the root system. Epicormic shoots, excised or micro-grafted shoot apices (0.2–0.4 mm), can also be used (see Notes 7 and 8, Fig. 1).
2. Upon collection, maintain soft ramets in suitable humid conditions, wrapped for instance in moistened tissue paper and placed in cool boxes to avoid hydric stress until utilization in the shortest delays (see Note 9).
3. Cut shoots into small pieces of 2–3 cm long with at least one node. Wash under running tap water for several minutes.
4. Soak explants in 70% ethanol for 5 min, followed by immersion in 1 mg/L  $\text{HgCl}_2$  (see Note 5) for 3 min, and 3 rinses in sterile distilled water.

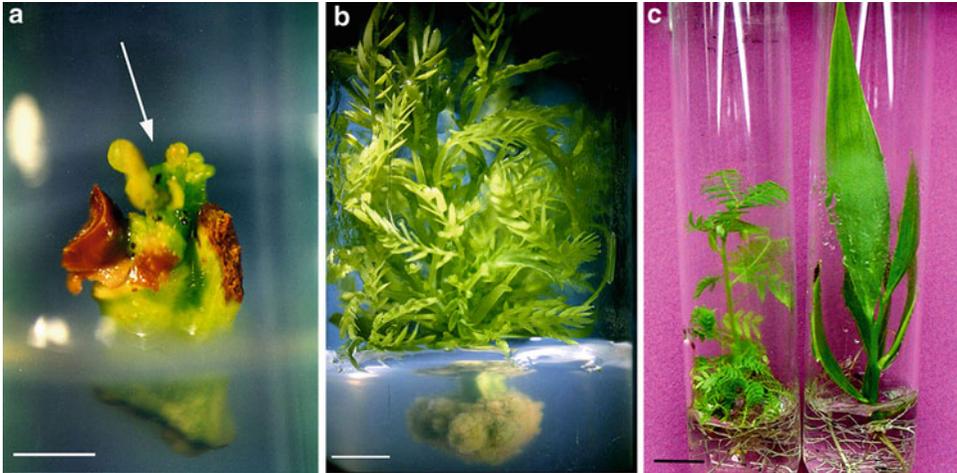


Fig. 2. Micropropagation of mature selected *A. mangium* genotypes. (a) Primary culture using a monodonal explant with an elongating axillary bud (arrow); (b) well-established explant showing a lot of axillary shoots with juvenile morphology; (c) in vitro rooted microshoots from the same mature clone exhibiting the compound-leaf juvenile morphology (left) and the phyllode mature morphology (right). Scale bars correspond to 0.5 mm for a and b, and 1 cm for c.

5. Cut upper and basal parts of the nodes to obtain 1 cm-long single node explants with a longer portion of segment below than above the node. This is done using scalpels on sterilized pieces of absorbing paper, under the laminar flow hood.
6. Culture explants vertically and individually in the tubes by digging 1–3 mm basal portion of the explant into the culture medium (see Figs. 2a and 3a), and transfer them to the culture room under above mentioned conditions for further development (see Note 10).

### 3.3. Developing and Maintaining the Cultures

1. After 2 months of primary culture, the base of every explant must be refreshed by a basal cut (see Note 11).
2. Trim the newly regenerated shoots in 1 cm-long microcuttings with at least one node or terminal bud, then transfer these microcuttings to tubes (1 or 2 per tube) containing fresh basal culture medium (see Note 12).
3. Place the newly inoculated tubes under the standard culture room conditions (16 h photoperiod and  $26 \pm 2^\circ\text{C}$ ).
4. The subcultures must be carried out regularly after every 2–2.5 month interval (see Note 13). As multiplication rate of the cultures increases, the test tubes can be replaced by suitable glass jars for higher mass production and cost efficiency (see Note 14; Fig. 3b, c). Cultures can be sustainably maintained using this protocol for years (see Note 15). Practical multiplication rates by axillary budding range between 3 and 5 at the end of every 2 month-long subculture (see Note 16).



Fig. 3. Micropropagation of mature selected *A. mangium* × *A. auriculiformis* genotypes. (a) Primary culture using a mononodal explant, already rooted with an elongating axillary bud (arrow, scale bar = 1 cm); (b, c) mass production in flasks at YSG Biotech, Sabah, Malaysia; (d) ex vitro acclimatization under mist system facilities in SSSB, Sabah, Malaysia and (e) under shade-cloth in Vietnam; (f) in vitro rejuvenated *A. mangium* × *A. auriculiformis* cloned stock plants used for mass producing rooted cuttings at lower cost under appropriate nursery conditions in Vietnam.

**3.4. Adventitious Rooting and Acclimatization to Ex Vitro Conditions**

1. Almost all *A. mangium* × *A. auriculiformis* shoot explants root on the basal medium and standard light conditions.
2. For *A. mangium*, 2 cm-long shoot tip explants, trimmed from axillary shoots developed on the multiplication medium are used for rooting.
3. Insert the base of these 2 cm-long shoot tip explants into the rooting medium contained in each of the 21 × 150 mm glass culture tubes covered with polypropylene caps (see Note 3).
4. Cultures are maintained in total darkness for 3 weeks before transferring them back to the standard conditions, i.e., 16 h photoperiod (50–60 μmol m<sup>2</sup>/s) at 26 ± 2°C.
5. The microshoots, showing newly formed elongating roots after 2–3 weeks (32), are pulled out from the culture tubes and the gelling agent sticking to the roots is carefully removed by washing under running tap water before transferring to ex vitro conditions (see Note 17).

6. Insert the rooted base of the microshoots in the sand bed (see Notes 18 and 19; Fig. 3d, e) in the nursery under high humidity (see Note 20) and natural light exposure, but avoiding direct sun irradiance by the utilization of shade screens (30–50% of shade) made of various materials (polypropylene cloth, see Fig. 3e).
7. After 3 or 4 weeks, the humidity level is gradually lowered.
8. The developing microshoots are progressively acclimatized to the outdoor conditions. The new roots will become more adapted to natural substrate than those formed in gelled tissue culture media.
9. At this stage, the rooted microshoots can be potted individually in 10×15 cm containers filled with clay top soil, mixed with some sand, avoiding hydric stress and direct sun exposure during the first weeks.
10. After 3 months of development under these nursery conditions, the plants reach a suitable size to be field planted (Fig. 3f).

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#### 4. Notes

1. For seed germination, the medium composition is half-strength MS macro- and micronutrients and 20 g/L sucrose. Suitable media for the subsequent steps of micropropagation and rooting from in vitro germinated seedlings are detailed in the literature (28). For producing seedlings to be used as in vitro rootstocks for micrografting, pour 5 mL of this liquid medium into the 21×150 mm glass test tubes, containing the plugs used as supports (30).
2. Culture media are prepared using the following concentrated stock solutions: macronutrient (×10), iron (×20), micronutrients (×1,000), vitamins (×1,000), myo-inositol (×100), growth regulators (×1,000). These stock solutions are stored at 2–4°C in darkness for up to 6 months. For more details, see ref. (33).
3. Once the cultures are successfully established, replace test tubes with glass jars (300–800 mL as total volume) and cover them with appropriate caps for higher mass production efficiency.
4. After sterilization, store culture media for a limited period preferably in the darkness at low temperature (2–4°C).
5. Addition of a few drops of a wetting agent (dishwashing soap more easily accessible and cheaper than Tween 20 or 80) increases the efficiency of the disinfecting solution.
6. As for a lot of arborescent species, seed germination rate in *Acacia mangium* may vary, usually between 60 and 70%.

7. Epicormic shoots can be produced by low branch portions of 60–70 cm long, collected from the selected trees, and placed horizontally on sand bed under mist system. Felling the selected trees in order to expect the development of sprouting shoots from the stump is another option (21, 34) but more destructive and risky because the stump may fail to sprout, and as a result, the genotype may be lost.
8. Another option for initiating *Acacia* spp. tissue culture is to use shoot apices (0.2–0.4 mm) further to their in vitro micrografting onto 2- to 3-month-old rootstocks produced from seedlings germinated and tissue cultured in vitro on plugs saturated with suitable liquid medium (see Fig. 1). This innovative technique (35) is useful for avoiding contaminations, particularly the endogenous ones (*Acacia* spp. develop naturally and synergically with *Bradyrhizobium* bacteria), and for inducing varying degrees of physiological rejuvenation (30). In vitro micro-grafted materials can beneficially be used for further mass micropropagation or for outdoor plant production. For *A. mangium* mature selected genotypes, success rate is far higher for in vitro micrografting than for more conventional in vivo grafting (35). On the other hand, shoot apex (0.1–0.2 mm as overall size) cultures from mature outdoor selected *A. mangium* trees on gelled media have been attempted but failed to develop into microshoots (Monteuuis, unpublished results). Despite being reported for *A. mearnsii* (34), meristem culture per se seems not possible due to the minute size of *Acacia* spp. shoot apical meristem (30  $\mu\text{m}$  in height for 90  $\mu\text{m}$  in diameter as average measurements for *A. mangium*).
9. The identity and origin of the selected plant material to be micropropagated must be recorded and labeled properly and with great care using practical and reliable means, such as permanent markers and pencil, thereby avoiding risks of mixing-up distinct origins.
10. Placing the primary explants in the darkness for the first 4–7 days following the in vitro introduction is advisable for excised shoot apex explants.
11. Contaminated explants are checked and promptly removed on a daily basis.
12. Trimming and transfer of explants is done in contamination-free conditions under laminar flow hood, on sterilized absorbing filter paper, or newspapers to save costs, using scalpels and forceps which are flamed after dipping in 95% ethanol.
13. The explants, especially taken from mature trees, gradually become more organogenic; thus the multiplication rate increases. The morphology may revert to juvenile-like form with compound leaves instead of the phyllode type that characterizes the mature stage (see Fig. 2b, c). Spontaneous rooting can even be observed at this stage.

14. Test tubes containing only one explant are preferably used during the first stages of micropropagation in order to limit the expansion of contaminations that may arise from the introduction in vitro of primary explants taken from the greenhouse or field-grown plant material.
15. These protocols have permitted to micropropagate sustainedly *A. mangium* and *A. mangium* × *A. auriculiformis* mature clones for more than 20 years up to now, which is more than the usual life span of such trees in natural conditions, and these materials are still being micropropagated with the same responsiveness.
16. These figures, quite conservative (9), are based on records established during several years for different *A. mangium* mature genotypes. They include possible contamination or physiological mortality losses, quantitatively quite limited overall. We deliberately chose to use moderate concentration of BA to ensure sustainable long-term micropropagation using a sole culture medium suitable for shoot elongation and multiplication by axillary budding. This seems more natural and safer for preserving the true-to-typeness of the materials, while reducing the risks of somaclonal variations.
17. Rooting rates are usually higher than 80% for juvenile materials and can reach 70% for mature selected genotypes (18, 29, 32).
18. River sand, more or less chemically neutral and pathogen-free, is recommended as a potting substrate. It is advisable to boil it before use and to apply efficient soil fungicides in aqueous solutions. This sand must be fine enough, but not overly for ensuring a good drainage.
19. Since *Acacia* spp. roots are very fragile, shorter roots reduce the risks of damage and breakage. Usually a pre-hole is made for the insertion of the rooted microshoots into the sand, thereby reducing the risks of breaking the newly formed roots.
20. High relative humidity (more than 80%) is usually provided by a reliable automatic mist-system.

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## In Vitro Propagation of Ash (*Fraxinus excelsior* L.) by Somatic Embryogenesis

Maurizio Capuana

### Abstract

Induction of somatic embryogenesis is described in common ash (*Fraxinus excelsior* L.). Embryogenic tissues are obtained from immature zygotic embryos and cultured on a modified Murashige and Skoog (MS) medium containing 8.8  $\mu\text{M}$  2,4-dichlorophenoxyacetic acid and 4.4  $\mu\text{M}$  benzyl-adenine. Embryogenic tissue is subcultured and multiplied on medium supplemented with reduced concentration of plant growth hormones. Somatic embryos develop and mature by transfer to hormone-free medium and subsequent culture on medium containing low amount of benzyladenine. Somatic embryo germination and conversion are enhanced by cold storage at 4°C and successive transfer onto Woody Plant Medium (WPM). Fully developed plantlets are then transferred to pots and acclimatized in the greenhouse equipped with a mist system.

**Key words:** Cold storage, Organogenesis, Plant growth regulators, Somatic embryogenesis, Zygotic embryo

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### 1. Introduction

More than 60 species of hardwood trees and shrubs belong to the genus *Fraxinus*. Among them, common ash (*Fraxinus excelsior* L.) and narrow-leaved ash (*F. angustifolia* Vahl.) are highly valuable hardwood tree species in several European countries for wood production and landscaping. Flowering ash (*F. ornus*) has a smaller distribution and is available in some South European countries, mainly Spain, Italy, Austria, and Balkan region. Important North American species include white ash (*F. americana*) and green ash (*F. pennsylvanica*). All these species are also popular as ornamental trees, used in urban forestry and private gardens. Particularly appreciated are some ornamental forms of *F. excelsior*, such as weeping ash (cv. *Pendula*), golden ash (cv. *Jaspidea*), and single-leaf ash (cv. *Diversifolia*).

The continuous selection of ornamental ash types with superior phenotypic traits for ornamental is carried out regularly. Vegetative propagation of selected superior trees is quite crucial for wide distribution. Conventional vegetative propagation of ash, such as cutting propagation (1) and grafting onto seedlings, may be applied, however have some limitations. Cutting propagation method presents some difficulties in achieving consistent rooting results, which are related to the rooting potential of the different genotypes, particularly when dealing with mature trees (2). Grafting is also a laborious and costly practice (3). Ash species can be multiplied in vitro by micropropagation methods such as axillary shoot micropropagation, adventitious shoot organogenesis, and somatic embryogenesis. These methods have been applied by culturing explants of different ages and origins, even though early reports have been with juvenile material (4–8).

Somatic embryogenesis has been obtained in *F. angustifolia* (9), *F. americana* (10), and *F. excelsior* (11). This is the most promising method for clonal mass propagation of trees, due to its enormous potential in terms of productivity and possibility of automation (12, 13). It also provides a useful tool for applications in clonal forestry allowing a multiclonal plantation combined to genetic test showing which clones are the best performers (14), in genetic engineering programs (15, 16) and for long-term germplasm conservation by cryopreservation (17, 18). This manuscript describes a protocol for somatic embryogenesis induction from zygotic explants of *F. excelsior* with subsequent somatic embryo maturation and conversion.

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## 2. Materials

### 2.1. Preparation of Culture Media

1. Murashige and Skoog salts (MS) (19) and Woody Plant Medium salts (WPM) (20) (Table 1).
2. D-sucrose pure.
3. Gelrite (Merck and Co., USA) and Agar (B&V, Italy).
4. 2,4-dichlorophenoxyacetic acid (2,4-D).
5. N<sub>6</sub>-benzyl-adenine (BA).
6. KOH solutions 0.1 and 1.0 M.
7. HCl solutions 0.1 and 1.0 M.
8. Glass flasks 125, 500 mL.
9. Beakers 500, 1,000 mL.
10. Cylinders 500, 1,000 mL.
11. Sterile Petri dishes (6 cm in diameter).
12. Test tubes.

**Table 1**  
**Plant culture media, based on the formulations of Murashige and Skoog ( $\frac{1}{2}$ MS, MS, 19) and Lloyd and McCown (WPM, 20)**

	$\frac{1}{2}$ MS <sup>a</sup>	MS <sup>b</sup>	WPM <sup>c</sup>
	mg/l	mg/l	mg/l
KNO <sub>3</sub>	950	1,900	–
NH <sub>4</sub> NO <sub>3</sub>	825	1,650	400
MgSO <sub>4</sub> ·7H <sub>2</sub> O	185	370	370
KH <sub>2</sub> PO <sub>4</sub>	85	170	170
CaCl <sub>2</sub> ·2H <sub>2</sub> O	220	440	96
Ca(NO <sub>3</sub> ) <sub>2</sub> ·4H <sub>2</sub> O	–	–	556
K <sub>2</sub> SO <sub>4</sub>	–	–	990
H <sub>2</sub> BO <sub>3</sub>	6.2	6.2	6.2
MnSO <sub>4</sub> ·4H <sub>2</sub> O	22.3	22.3	22.3
ZnSO <sub>4</sub> ·7H <sub>2</sub> O	8.6	8.6	8.6
Na <sub>2</sub> MoO <sub>4</sub> ·2H <sub>2</sub> O	0.25	0.25	0.25
CuSO <sub>4</sub> ·5H <sub>2</sub> O	0.025	0.025	0.25
CoCl <sub>2</sub> ·6H <sub>2</sub> O	0.025	0.025	–
KI	0.83	0.83	–
FeSO <sub>4</sub> ·7H <sub>2</sub> O	27.8	27.8	27.8
Na <sub>2</sub> EDTA·2H <sub>2</sub> O	37.3	37.3	37.3
Sucrose	20,000	20,000	20,000
Glycine	2.0	2.0	2.0
Pyridoxine·HCl	0.5	0.5	0.5
Nicotinic acid	0.5	0.5	0.5
Thiamine·HCl	0.1	0.1	1.0
Myo-inositol	100	100	100

<sup>a</sup>Induction medium

<sup>b</sup>Maturation medium

<sup>c</sup>Conversion medium

13. Tissue culture facilities: scalpels, forceps, sterilizer, precision balance, magnetic stirrer, magnetic bars, microwave cooker, pH meter, autoclave for sterilization, laminar flow bench, growth chamber, refrigerator.

### **2.2. Sterilization of the Explants**

1. Tap water.
2. Ethanol (70 %).
3. Sodium hypochlorite solution (bleach solution at 7 g/L active chlorine).
4. Distilled water (autoclaved reverse-osmosis water).
5. Sterilized glass flasks 125, 250 mL.
6. Cylinders 50, 100, 250 mL.
7. Tissue culture facilities: forceps, sterilizer, laminar flow bench, growth chamber, refrigerator.

### **2.3. Induction and Proliferation of Embryogenic Tissues; Plantlet Formation**

1. Tissue culture facilities: precision balance, magnetic stirrer, magnetic bars, microwave cooker, pH meter, autoclave for sterilization, laminar flow bench, refrigerator.

### **2.4. Acclimatization of Plantlets**

1. Plastic trays (with 3.5 cm diameter holes).
2. Plastic pots (8 cm diameter).
3. Potting medium (garden soil, peat, sand 3:1:1 by volume).
4. Greenhouse equipped with “mist” system.

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## **3. Methods**

In majority of woody species, immature zygotic embryo explant has been the most successful explant to induce somatic embryogenesis. By using this starting material, clonal propagation does not replicate a defined genotype; however, it can initiate clonal propagation from material obtained by controlled crosses of superior genotypes, which are of remarkable importance. The following protocol comprises seven stages: (1) preparation of culture media; (2) plant material collection and sterilization; (3) induction of somatic embryogenesis; (4) maintenance and proliferation of embryogenic tissues; (5) somatic embryo maturation and germination; (6) somatic embryo conversion; and (7) acclimatization of plantlets.

### **3.1. Culture Media and Conditions**

1. Prepare MS (full-strength) and  $\frac{1}{2}$  MS (half-strength MS macro-elements, full-strength MS micro-elements, iron, and vitamins—Table 1) media in doubled distilled water, supplemented with 2 % sucrose. Store at 4°C.
2. Prepare 2,4-D and BA stock solution: 2,4-D must be dissolved in a few drops of absolute ethanol; for BA use 0.1 M KOH. Store at 4°C.

3. Induction medium: use ½ MS supplemented with 8.8 µM 2,4-D and 4.4 µM BA.
4. Proliferation medium: use plant growth regulator (PGR)-free ½ MS medium.
5. Maturation medium: use PGR-free MS medium (Table 1).
6. Germination medium: use MS medium containing 0.44 µM BA.
7. Adjust the pH of the media to 5.8 using HCl or KOH (1.0 and 0.1 M).
8. Add gelrite 0.36 %.
9. Sterilize the media by autoclaving at 121°C and 108 kPa for 20 min.
10. Store the autoclaved media at 4°C for a maximum of 60 days.

### **3.2. Source Material; Surface Sterilization**

1. Collect seeds from the selected plant(s) and store them at 4°C until use (see Note 1).
2. Discard the external coats (wings) of seeds and rinse the seeds under slow running tap water for 24 h.
3. Sterilize the laminar flow surface by 70 % ethanol before use.
4. Disinfect the seeds by soaking in 70 % ethanol solution for 2 min, followed by two 2-min rinses in sterile distilled water; disinfect again by soaking in 20 % sodium hypochlorite (1.4 % active chlorine) solution for 20 min, with three final rinses in sterile distilled water, under the laminar air flow and using sterilized glass flasks.

### **3.3. Somatic Embryogenesis Induction**

1. Under the laminar flow bench, open the seed with the scalpel, pick up the embryo, and culture in the initiation medium (12 embryos per Petri dish) (see Note 2).
2. Incubate the cultures in the growth room (or cabinet) in darkness.
3. After one month, transfer the explants onto fresh induction medium.

### **3.4. Embryogenic Tissue Maintenance and Proliferation**

1. Transfer explants with emerging embryogenic tissues from the induction medium to a PGR-free ½ MS medium (see Note 3) (Fig. 1a).
2. Incubate cultures at 16 h photoperiod light condition, 20 µmol/m<sup>2</sup>/s photosynthetically active radiation, for 1 month.
3. Subculture the material at 2-week interval on MS medium amended with 0.44 µM BA.

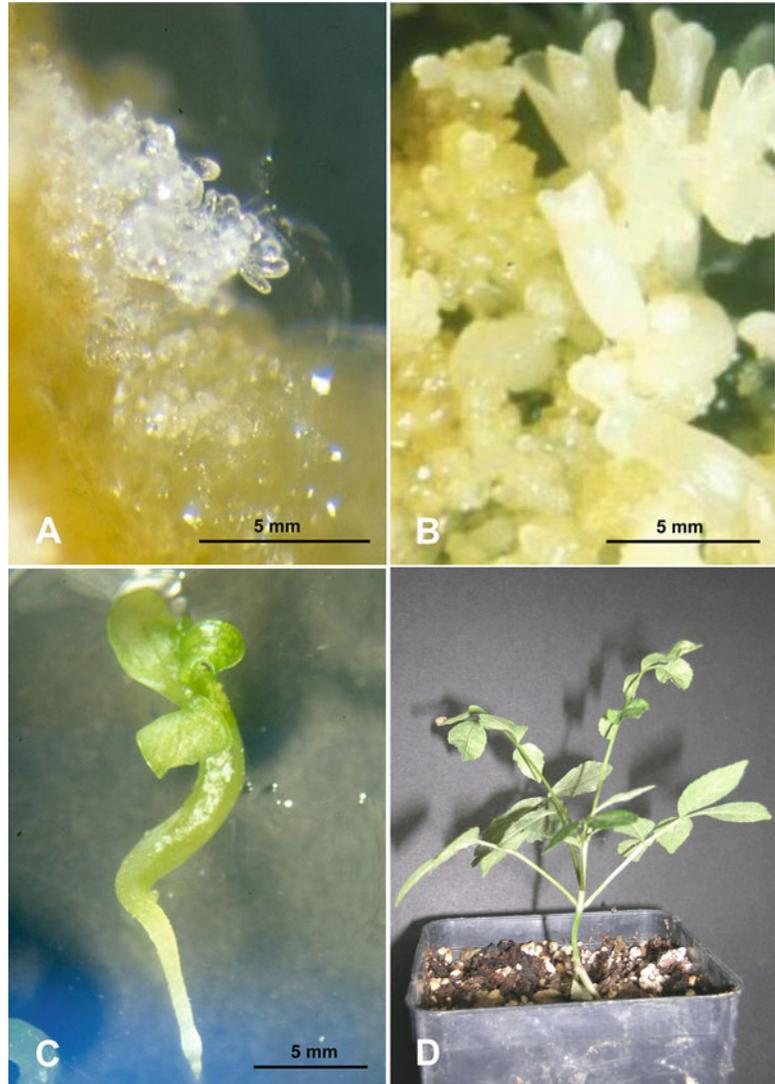


Fig. 1. (a) Embryogenic tissues growing from the original explant (immature zygotic embryo), (b) cluster of maturing somatic embryos, at stages from globular to cotyledonary, (c) germinating somatic embryo, (d) plant growing from somatic embryo, 8 months after transfer to pot and acclimatization.

### **3.5. Somatic Embryo Maturation and Germination**

1. Transfer embryogenic tissues on PGR-free MS medium for 4-week culture duration (see Note 4).
2. From clusters of maturing somatic embryos (just before the developmental stage showed in Fig. 1b), isolate globular embryos (singularization) and culture them for four weeks on MS medium containing  $0.44 \mu\text{M}$  BA (see Notes 5 and 6).

### **3.6. Somatic Embryo Conversion**

1. Transfer somatic embryos at cotyledonary stage onto Petri dishes containing PGR-free MS medium and store for three weeks at  $4^{\circ}\text{C}$ , in the darkness (see Note 7).

2. Subculture on WPM medium (Table 1) containing 0.44  $\mu\text{M}$  BA and incubate at light 40  $\mu\text{mol}/\text{m}^2/\text{s}$  photosynthetically active radiation (see Note 8) (Fig. 1c).

### 3.7. Acclimatization

1. Select the plantlets with a shoot length of about 2 cm and wash them under running tap water to remove the adhering solidified culture medium.
2. Insert the plantlets in 35 mm diameter trays filled with potting mixture. The trays are placed on a greenhouse bench equipped with a mist system for 2 weeks.
3. For a gradual transition to ambient atmosphere, move the trays to a nonmisted bench under a tunnel covered with plastic foil, where they remain for about 3 weeks. At this stage, the plantlets show a well-developed root system and at least 3–4 fully open leaves.
4. Transplant the plantlets to larger pots (80 mm diameter) and place the pots in a shaded area of the nursery for further growth (Fig. 1d) (see Note 9).

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## 4. Notes

1. The initiation of somatic embryogenesis is greatly dependent on the type of explant used. Zygotic embryos are responsive to embryogenic induction only for a short period of time during their maturation. *F. americana* has a different behavior, because only mature zygotic embryos proved to be suitable for somatic embryogenesis (21). *F. excelsior* seeds are collected during their maturation on the tree, when zygotic embryo begins to show initial development of cotyledons and the endosperm starts to solidify (early July, at South European latitude). At this stage, seeds generally show bright green external coat and absence of yellow/brown dots. When seeds are collected earlier, they generally give no morphogenetic response. In seeds sampled at more advanced stages of embryo development, only organogenetic phenomena are observed.
2. In *F. angustifolia* (9), somatic embryogenesis is obtained when nutrient media contain an auxin–cytokinin ratio 2:1. Induction frequency of somatic embryogenesis, however, is rather low as compared to organogenesis, which consists more frequently in the production of shoots; some root primordials can also be observed.
3. Embryogenic tissues normally continue to proliferate after transfer to PGR-free medium, where a sporadic and asynchronous development of somatic embryos to cotyledonary stage

can be observed. The embryogenic tissues can be subcultured for years without showing any loss of proliferation capacity, but after some months of culture on PGR-free medium the proliferation of embryogenic tissues starts to decline, suggesting the transfer of cultures onto a BA-containing medium.

4. The use of other combinations of mineral media and PGR content can have different influences on the tissue growth; for instance, WPM or Driver and Kuniyuki Walnut (DKW) (22) media containing 0.44  $\mu\text{M}$  BA promote a faster embryo maturation, while WPM with no PGR induces some necrosis of tissues (11).
5. Somatic embryos germinate on PGR-free medium, but a faster development of epicotyl is achieved on the medium containing 0.44  $\mu\text{M}$  BA. Moreover, to keep a small amount of cytokinin in the subculturing medium helps to avoid the appearance of stress symptoms (necrosis of the apical pole, prevalently) noticeable after the fourth to fifth week of culture on PGR-free medium. On an average, 80 % somatic embryos can germinate. Many somatic embryos may show supernumerary or fused cotyledons; such abnormalities, however, not always hamper the capacity of embryos to germinate and convert into whole plantlet. In *F. angustifolia*, 1-month subculture of embryogenic tissues on medium containing 30 g/L mannitol and 2 g/L activated charcoal helps to optimize somatic embryo maturation and prevention of early germination (9, 23).
6. Exposure of globular-stage somatic embryos to 3.8  $\mu\text{M}$  abscisic acid (ABA) results in a faster development of the apical pole (24), while the rooting apex show the best growth after culture on PGR-free medium (Fig. 1c). ABA-treated somatic embryos show an altered growth, with partial vitrification of shoots (11).
7. The conversion of somatic embryos is consistently enhanced by chilling treatments (11, 23).
8. Somatic embryo conversion is successful on either MS or WPM media, but with the latter medium the growth of root results faster. Plantlets developed from somatic embryos generally have normal growth and appearance.
9. By this procedure 80 % plantlets can be successfully acclimatized.

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## Micropropagation of Paradise Tree (*Melia azedarach*) by In Vitro Culture of Axillary Buds

Luis A. Mroginski and Hebe Y. Rey

### Abstract

Paradise tree (*Melia azedarach* L.) is a multipurpose ornamental and timber tree, and its extracts are used to make insecticides and fungicides. Conventional propagation is done by seeds; however, sexual reproduction results in wide genetic variability. Therefore, clonal propagation is desirable to reduce genetic variation. This chapter describes a protocol for in vitro propagation of paradise tree by axillary buds. There are major steps for this protocol. Firstly, shoot induction by in vitro culture of axillary buds, excised from potted plants obtained by rooting of cuttings of 10–15-year-old adult trees. The initiation medium was composed of Murashige and Skoog medium (MS) supplemented with 0.5 mg/L BAP (benzylaminopurine), 0.1 mg/L IBA (indolebutyric acid), and 0.1 mg/L GA<sub>3</sub> (gibberellic acid). Secondly, multiplication of the regenerated shoots on MS medium amended with 0.5 mg/L BAP and 0.1 mg/L GA<sub>3</sub>. Thirdly, rooting of the regenerated shoots on MS medium containing 0.1 mg/L IBA. Fully well-developed plants were transferred to pots containing sand, peat moss, and perlite (1:1:1), and maintained initially in the greenhouse or plastic tunnels.

**Key words:** Axillary buds, *Melia azedarach*, Micropropagation, Tissue culture

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### 1. Introduction

Paradise tree (*Melia azedarach* L.—Meliaceae) is a native species of the Southern Asia—Iran, India, and South of China. It was introduced in the New World and spread throughout tropical America, from Mexico to Argentina (1). This tree is well adapted to different types of soils and climatic conditions and highly resistant to insects (2). Paradise tree is a multipurpose ornamental tree species and several useful products are extracted from different parts of the plant (leaves, stems, seeds, flowers, and fruits) for use as insecticide (3–7) and fungicide (8). The extract of leaves has an antiviral factor that inhibits the replication of several viruses (9, 10). In the Province of Misiones, Argentina, the paradise tree is cultivated as forest (11).

Paradise trees are readily propagated by seeds and create wide genetic variation (12–14). Therefore, its clonal propagation is highly desirable by using in vitro culture as an alternative method of propagation and also produce in large numbers from small pieces (explants) in a relatively short period. Different aspects of the tissue culture of *M. azedarach* has already been carried out on plant regeneration by indirect organogenesis from leaf explants (15, 16) and roots (17), and through somatic embryogenesis (18–20). Plant regeneration was also reported from both axillary bud culture and nodal explants (12–14) as well as from shoot apical meristems (21). In this report, a protocol is described for shoot induction from axillary buds, with subsequent rooting and acclimation of regenerated plants.

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## 2. Materials

### 2.1. Source of Material and Surface Sterilization

1. Potted plants as source of axillary buds.
2. Growth room at  $27 \pm 2$  °C under a 14 h photoperiod provided by cool-white fluorescent lamps, with an irradiance of  $4.5 \mu\text{mol}/\text{m}^2 \text{ s}$ .
3. Sterile distilled water.
4. Ethanol 70% (v:v).
5. Commercial bleach solution (containing NaOCl, 2% active Cl).
6. TRITON X-100® 0.1% (Merck, Darmstadt, Germany).
7. Sterile glass or plastic Petri dishes (9 cm diameter).
8. Tissue culture facilities (laminar flow bench, pH meter, autoclave), instruments (forceps, scalpel, needles, razor, and blades).

### 2.2. Culture Media

1. Stock salt formulations from Murashige and Skoog medium (22) for (a) establishment of bud cultures, (b) shoot multiplication, and (c) root induction. Media compositions are given in Table 1.
2. Agar (A-1296 from Sigma Chemical Co).
3. 0.1 N HCl and 0.1 N KOH to adjust pH of the culture medium.
4. Glass test tubes (11 mL capacity) and glass jars (300 mL capacity).
5. Aluminum foil and Resinite® (Casco S. A. C. Company, Buenos Aires) film.

### 2.3. Acclimation of Regenerated Plants to Ex Vitro Conditions

1. Plastic pots (8 cm diameter) containing sand, peat moss, and perlite (1:1:1).
2. Tap water.
3. Transparent plastic bags (12 cm diameter).

**Table 1**  
**MS medium formulation according**  
**to Murashige and Skoog (22)**

Components	(mg/L)
$(\text{NH}_4)\text{NO}_3$	1.650
$\text{KNO}_3$	1.900
$\text{KH}_2\text{PO}_4$	170
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	370
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	440
KI	0.83
$\text{H}_3\text{BO}_3$	6.20
$\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$	22.30
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	8.60
$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	0.25
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.025
$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	0.025
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	27.80
$\text{Na}_2\text{EDTA}$	37.30
Glicine	2.00
Tiamine-HCl	0.1
Piridoxine-HCl	0.5
Nicotinic acid	0.5
Myo-inositol	100
Sucrose	30.000

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### 3. Methods

The micropropagation of Paradise tree through direct organogenesis by in vitro culture of axillary buds involves three steps and one final step of acclimation of the regenerated plants to the ex vitro conditions. Firstly, in vitro step comprises of the selection of plant material and the establishment of cultures of axillary buds using the initiation medium. Second step includes the induction of multiple buds and shoots, and the maintenance of the cultures by using a multiplication medium. Third step is the rooting of regenerated shoots by using a rooting medium. Finally, the regenerated plants need a period of acclimation to ex vitro conditions. Using this protocol, the regenerated plants of paradise tree appear morphologically similar to the mother plants.

### **3.1. Preparation and Sterilization of the Culture Media**

1. Prepare MS medium according to the compositions given in Table 1.
2. For preparation of MS medium, the chemicals (sucrose, mineral nutrients, and vitamins) are dissolved in distilled water. It is recommended to prepare ten times more concentrated (MS  $\times 10$ ) and store in a freezer, in plastic bags of different capacities (10, 100, 500 mL, according to the requirement).

To prepare MS  $\times 10$ :

- (a) Dissolve macro salts (Table 1) in 300 mL distilled water.
  - (b) Add 10 mL  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  solution (44 g/100 mL water).
  - (c) Add 10 mL of a solution composed of 3.73 g of  $\text{Na}_2\text{EDTA}$  and 2.78 g of  $\text{Fe SO}_4 \cdot 7\text{H}_2\text{O}$ . This solution must be stored in refrigerator.
  - (d) Add 10 mL KI solution (83 mg/100 mL water). This solution must be stored in amber bottle in refrigerator.
  - (e) Add 10 mL micro-salt stock solution (Table 1), prepared in 100 mL water, stored in the refrigerator.
  - (f) Add 10 mL vitamin stock solution (Table 1), prepared in 100 mL water, stored in the refrigerator.
  - (g) Add 30 g sucrose.
  - (h) Adjust the volume to 1,000 mL with distilled water.
3. Preparation of initiation medium: MS medium supplemented with 0.5 mg/L BAP (benzylaminopurine), 0.1 mg/L IBA (indolebutyric acid), and 0.1 mg/L  $\text{GA}_3$  (gibberellic acid), add 100 mL MS  $\times 10$ , 5 mL BAP solution (100 mg/L), 1 mL IBA solution (100 mg/L), 1 mL  $\text{GA}_3$  solution (100 mg/L), and adjust the volume to 1,000 mL with distilled water. Adjust the pH of the medium to 5.8 using 0.1 N HCl or 0.1 N KOH. Add 0.8% (w:v) agar (A-1296 from Sigma Chemical Co). Dispense 3 mL initiation medium into glass test tubes (11 mL capacity). Sterilize the medium by autoclaving at 1.46 Kg/cm<sup>2</sup> for 20 min. Store the autoclaved medium in the darkness at 25 °C no more than 45 days.
  4. Preparation of multiplication medium: MS medium containing 0.5 mg/L BAP and 0.1 mg/L  $\text{GA}_3$ , add 100 mL MS  $\times 10$ , 5 mL BAP solution (100 mg/L), 1 mL  $\text{GA}_3$  solution (100 mg/L), and adjust the volume to 1,000 mL with distilled water. Adjust the pH of the medium to 5.8 using 0.1 N HCl or 0.1 N KOH. Add 0.8% (w:v) agar (A-1296 from Sigma Chemical Co). Dispense 50 mL multiplication medium into glass jars (300 mL capacity). Sterilize the medium by autoclaving at 1.46 Kg/cm<sup>2</sup> for 20 min. Store the autoclaved medium in the darkness at 25 °C no more than 45 days.

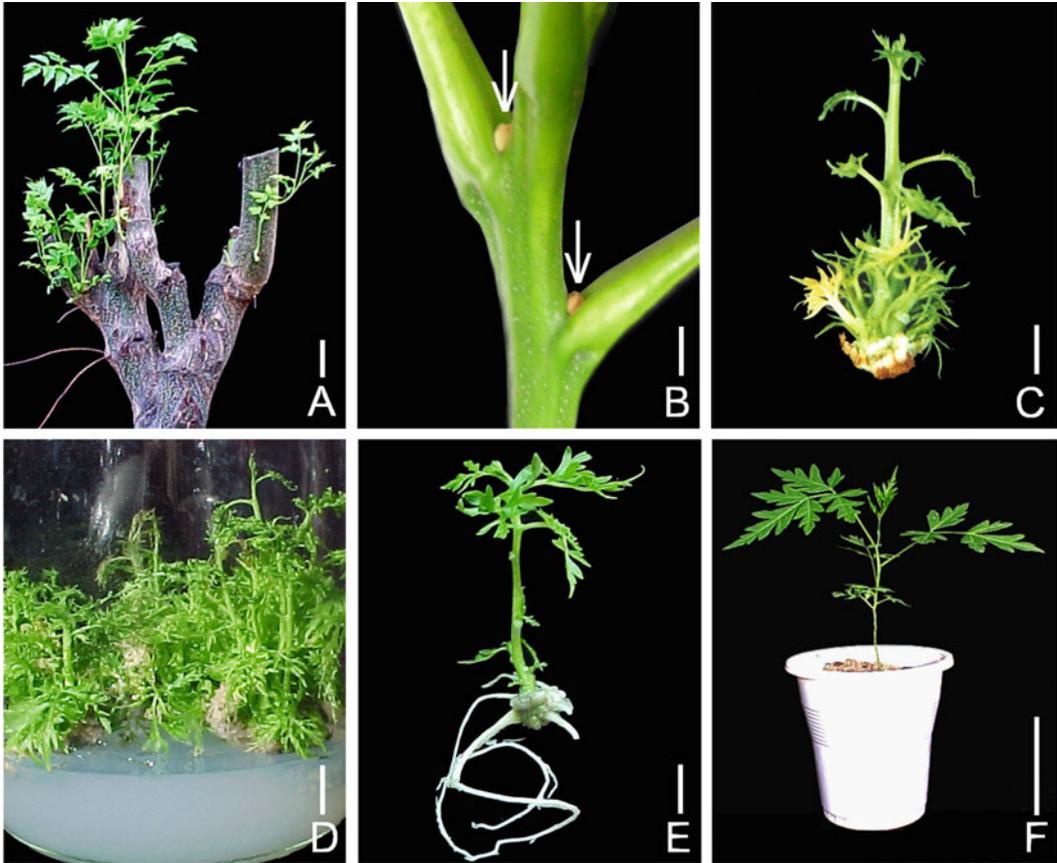


Fig. 1. In vitro plant regeneration from *Melia azedarach* L. axillary buds. (a): Young shoots, served as an explant source (bar, 4 cm), (b): Stem (arrows show the axillary buds cultured) (bar, 0.5 cm), (c): Shoot, regenerated after 4 weeks of culture on initiation medium (bar, 0.5 cm), (d): Multiple shoots after 4 weeks of culture on multiplication medium (bar, 0.5 cm), (e): Rooted shoot after 20 days of culture on rooting medium (bar, 0.5 cm), (f): Regenerated plant, growing in the greenhouse (bar, 4 cm).

5. Preparation of rooting medium: MS medium amended with 1 mg/L IBA, add 100 mL MS  $\times 10$ , 10 mL IBA solution (100 mg/L), and adjust the volume to 1,000 mL with distilled water. Adjust the pH of the medium to 5.8 using 0.1 N HCl or 0.1 N KOH. Add 0.8% (w:v) agar (A-1296 from Sigma Chemical Co). Dispense 50 mL rooting medium into glass jars (300 mL capacity). Sterilize the medium by autoclaving at 1.46 Kg/cm<sup>2</sup> for 20 min. Store the autoclaved medium in the darkness at 25 °C no more than 45 days.

### 3.2. Surface Sterilization of the Plant Material

1. Take young shoots (with approximately 4–6 leaves, Fig. 1a) from stock mother plants, obtained by rooting of cuttings, using the potting mixture described in Subheading 2.3, item 1, under the greenhouse conditions (see Note 1).
2. Wash shoots for 1 min with running tap water.

3. Remove all the leaves with a scalpel and surface sterilize stems by immersion in 70% ethanol (3 min), followed by 20 min immersion in a commercial bleach solution (containing NaOCl, 2% active Cl) with 0.1% TRITON X-100®. Finally, working in a laminar flow bench, wash the stems with abundant sterile distilled water and keep them (for approximately 1 h) in a sterile jar with distilled water until the dissection of the axillary buds.

### **3.3. Culture and Maintenance of the Explants (see Note 2)**

1. Excise axillary buds from surface sterilized stems (Fig. 1b) by using a sterile scalpel.
2. Place one axillary bud on the surface of the initiation medium, contained in the glass test tubes (11 mL capacity).
3. Cover the tubes with Resinite® film and incubate the explants in a growth room at  $27 \pm 2$  °C under a 14 h photoperiod provided by cool-white fluorescent lamps, with an irradiance of  $4.5 \mu\text{mol}/\text{m}^2 \text{ s}$ .
4. After 30 days of culture, shoots are formed in 40–70% of the cultures (Fig. 1c). Take one shoot (1–3 cm long with 3–5 leaves), discard the leaves and place the stem horizontally on 50 mL multiplication medium contained in a glass jar (300 mL capacity). Cover the tubes with Resinite® film and incubate the cultures in the same conditions described in the Subheading 3.3, type 3.
5. Transfer the regenerated shoots (Fig. 1d), without leaves, to a fresh multiplication medium in 30-day intervals. Repeat this transference for ten times (see Note 3).

### **3.4. Rooting of Regenerated Shoots In Vitro**

1. Excise well-developed shoots (as described in Subheading 3.3, type 4), regenerated in multiplication medium, and insert the bases of the stems into 50 mL rooting medium contained in 300 mL glass jars.
2. Cover the tubes with Resinite® film and incubate the cultures in the same conditions described in the Subheading 3.3, type 3.
3. After 20 days, regenerated shoots should develop root systems (Fig. 1e).

### **3.5. Acclimation of Regenerated Plants to Ex Vitro Conditions**

1. Remove the rooted shoots from the culture jars and carefully wash roots with tap water in order to remove culture medium sticking to roots.
2. Transfer each plant individually to plastic pots (8 cm diameter) containing sand, peat moss, and perlite (1:1:1) (see Note 4) and irrigate the plants thoroughly.
3. Insert the transparent plastic bags (12 cm diameter) on the top of the plants and transfer them to the greenhouse.
4. After 7 days, remove completely the plastic bags (Fig. 1f).
5. Plants show new growth that indicates acclimation of plants.

## 4. Notes

1. It is also possible to use selected young shoots, excised from field-grown trees, preferably during spring or summer. In this case, transport shoots in a plastic bag and use them immediately without any delay.
2. All maintenance operations of *in vitro* cultures should be carried out in laminar flow for preventing fungal and bacterial contamination, which is on an average 20–25%. Therefore, it is recommended to establish cultures in small test tubes, each containing single axillary bud. Avoid big jars, each cultured with several explants.
3. Our results show that the regenerated plants are no longer genetically identical to the mother plants after ten subcultures.
4. Regenerated plants of paradise tree can also be successfully transferred in other substrates including sand, vermiculite, and humus.

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## In Vitro Propagation of *Hydrangea* spp.

Barbara Ruffoni, Ermanno Sacco, and Marco Savona

### Abstract

*Hydrangea* (Hortensia) is a highly popular ornamental plant for garden decoration, and now it is commercially produced for cut flower branches. For in vitro culture, Murashige and Skoog medium supplemented with BA (0.25 mg/L) and sucrose (30 g/L) was used. Culture conditions were  $23 \pm 1^\circ\text{C}$  of temperature, light intensity of  $35 \mu\text{mol}/\text{m}^2/\text{s}$  P.P.F.D., and 16/8 h day/night photoperiod. Following shoot proliferation, the in vitro rooting frequency was 100% on a medium containing NAA 0.5 mg/L. However, 95% direct in vivo rooting was achieved by dipping microcuttings in a 5,000 ppm K-IBA solution which were transferred afterward to a glasshouse for acclimatization. After 21 days, fully acclimatized and well-established plants were obtained, suitable for commercialization. Furthermore, leaf fragments derived from in vitro plantlets were cultured for callus induction and adventitious shoot regeneration.

**Key words:** Hortensia, Micropropagation, BA, Rooting, K-IBA, Temporary Immersion System, Regeneration, Acclimatization

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## 1. Introduction

*Hydrangea* is a genus of about 75 species of flowering plants, native to Southern and Eastern Asia and North and South America. It belongs to the Hydrangeaceae family and most species are shrubs 1–3 m tall, but some are small trees and others lianas reaching up to 30 m high. The plant is better known internationally as “Hortensia” (1) and it is a very popular ornamental plant for garden decoration and commercial production of cut flower branches. *Hydrangeas* are renowned for their colorful and bountiful floral inflorescences that are spectacular spheres (corymbs or panicles) of different colors such as pink, blue, white, light purple, or dark purple. *Hydrangea* flowers are produced from early spring to late autumn. The inflorescences of many species contain two types of flowers: small fertile flowers in the middle and large sterile bract-like flowers in a ring around the edge of each sphere. The flower color often mirrors the pH of the soil: acidic soils produce blue

flowers, neutral soils produce very pale cream petals, and alkaline soils results in pink or purple. This change of flower pigments is due to presence of aluminum ions which can be taken up into hyper-accumulating plants. The plants, generally, have small-toothed dark green leaves on stiff-erect stems and medium-size flower heads.

Seed germination in *Hydrangea* spp. is not difficult (2–4); however, seedlings exhibit variability and do not always produce the desired characteristics or morphology (5). The clonal uniformity occurs by propagation with root cuttings, leading to the commercial propagation of *Hydrangeas* (5, 6). Little has been reported on the in vitro propagation and regeneration of *Hydrangea* spp. Tissue culture of *Hydrangea quercifolia* (7) showed the influence of thidiazuron (TDZ) on in vitro shoot proliferation (8), particularly cv Snow Queen (9). Several reports are available on micropropagation of *Hydrangea macrophylla* (9–11), and selected genotypes of *Hydrangea heteromalla* “Snow Cap” and *Hydrangea petiolaris* (9). Leaf regeneration was accomplished from leaves and internodes of *H. macrophylla* (12) and plant regeneration (11). Shoot organogenesis was successful from leaf explants of *H. macrophylla* “Hyd1” and the genetic stability of regenerates using ISSR markers was recently reported (13). This paper describes a protocol for in vitro propagation of *Hydrangea* spp., especially *H. quercifolia* “Snow Queen,” with the aim to increase the propagation potential of the plant produced either as a cut or a pot plant.

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## 2. Materials

### 2.1. Surface Sterilization of Source Material

1. Ethanol 70% prepared with 70 mL ethanol 99.8% and 29 mL distilled water.
2. Aqueous NaOCl solutions of active chlorine (e.g., commercial bleach ACE, Procter & Gamble, USA) plus two drops of surface-active agent Tween 20®.
3. Autoclaved reverse-osmosis water, 200 mL aliquots in 500 mL culture vessels.
4. Magnetic stirrer, 1,000 mL flask or beaker.
5. Tissue culture facilities: instruments (scalpel, forceps, spirit burner to flame sterilize instruments), laminar flow hood, culture room.
6. Potted plants *Hydrangea* spp. as a source of explants.

### 2.2. Culture Media

1. Media based on the formulation of Murashige and Skoog (MS; (14)) for shoot propagation from apical and axillary buds of young branches and root induction.
2. Glass culture vessels 500 mL with transparent caps.

3. Petri dishes (9 cm diameter, Bibby Sterilin, Stone, UK).
4. Parafilm (Parafilm® M Barrier Film, SPI. Supplies, West Chester, USA).
5. Duran glass flasks or beakers, 1,000 mL capacity (Schott AG, Mainz, Germany).

### **2.3. Temporary Immersion System**

1. RITA® (CIRAD, France) plastic containers.
2. Autoclavable plastic bags.

### **2.4. Acclimatization of Regenerated Plants to Ex Vitro Conditions**

1. Plastic alveolary pots.
2. Potting medium consists of peat (Trysubstrate Klasmann-Deilmann) and sterilized sand (1:1, v/v).

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## **3. Methods**

### **3.1. Preparation and Sterilization of Culture Media**

1. Dissolve MS (Duchefa Biochemie B.V., The Netherlands; cod M 0221.0010) powder (43 g), containing a micro- and macro-elements complex, in 9 L of deionized water to prepare a 10× stock solution; while stirring the water, add the powder and stir until complete dissolution; bring the solution to a final volume (10 L) by adding water; use 1 L of this solution (4,302.09 mg) for each liter of culture medium.
2. Dissolve MS vitamins (Duchefa Biochemie B.V., The Netherlands; cod M 0409.0250) powder, containing 25.8 g mixed vitamins, to prepare 250 mL of 1,000× stock solution in deionized water and stir until completely dissolved, eventually warming the solution up to 30°C. Use 1 mL vitamin stock solution (103.1 mg) for each liter of culture medium.
3. Add any desired stable supplement (commercial sucrose, usually 30 g/L) weighing the powder and dissolve by stirring.
4. Plant growth regulators stocks: to prepare a 1 mg/mL of stock solution, weigh 100 mg of the plant growth regulator 6-benzylaminopurine (BA), indole-3-butyric acid (IBA), indole-3-acetic acid (IAA), 1-naphthalenacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), kinetin (Kin), 2-isopentenyladenine (2-iP), and zeatin (Zea), and add in a volumetric flask 20–30 mL ethanol (99.8%). Gently stir until completely dissolved and bring slowly the volume to 100 mL with distilled water at room temperature. Store the stock solution as recommended (4°C in light for BA, IBA, 2,4-D, Kin, 2-iP, Zea, and NAA; 4°C in dark for IAA). Add the PGR as requested for each micropropagation phase.
5. After adding all the components, while stirring, adjust the pH of the medium (usually 5.7) using NaOH 1 N or HCl 1 M.

6. Add 8 g/L technical agar (we use a product of Duchefa Biochemie B.V., The Netherlands), heat until clarity of the solution, stirring the medium on a electric plate or heating in a microwave.
7. Dispense 62.5 mL medium into 16 culture vessels (500 cc).
8. Sterilize the medium in autoclave at 121°C, 1 atm (1.01325 bar) for 20 min. Allow medium to cool and solidify prior plant inoculation.
9. To pour culture medium in Petri dishes (9 cm diameter): follow instructions from steps 1 to 5, add 8 g/L technical agar, sterilize the medium in autoclave at 121°C, 1 atm (1.01325 bar) for 20 min, move the flask (when cooled below 100°C) under the laminar flow hood and dispense 25 mL medium in each Petri dish to prepare 40 Petri dishes.

### **3.2. Preparation of RITA® (CIRAD, France) Containers for TIS**

1. Fill the RITA® containers with 160 mL liquid medium.
2. Envelop separately each vessel with autoclavable plastic bag.
3. Sterilize in autoclave at 121°C, 1 atm (1.01325 bar) for 20 min.
4. Set the timer for 3 min, flooding every 3 h.

### **3.3. In Vitro Culture Establishment**

1. Excise apical and axillary buds of young branches of *Hydrangea* ornamental genotypes from mature greenhouse-grown plants, maintained at 20°C in 14-cm diameter pots, under natural light conditions; excising explants during April–May (northern hemisphere) produce better results (see Note 1).
2. Firstly, rinse explants in a detergent-warm aqueous solution with a few drops of liquid dish soap. Then, sterilize with 70% ethanol for 30 s, treat with NaOCl solution (5% active chlorine) for 15 min, and rinse twice with sterilized distilled water (see Note 2).
3. Transfer explants on the medium for in vitro culture establishment (Table 1) for 30 days.

### **3.4. Multiplication and Rooting**

1. Cut new buds and shoots from the *H. quercifolia* “Snow Queen” explants, discard the 2-mm explant base, transfer 12 shoots per vessel to the multiplication medium (Table 1) and subculture at every 30-day interval. Set the growth chamber conditions for the multiplication phase at 23 ± 1°C and 16 h photoperiod (light intensity 35 µmol/m<sup>2</sup>/s P.P.F.D.). Calculate the multiplication rate as number of shoots per explant per month at the end of each subculture (see Note 3).
2. Transfer 12 single shoots per RITA® containers for Temporary Immersion System (TIS) (see Subheading 3.2) containing agar-free multiplication medium (Table 1), and set the growth

**Table 1**  
**Media composition for in vitro propagation of *Hydrangea* ornamental genotypes**

	In vitro establishment	Multiplication	Rooting
MS salts (L)	1	1	1
MS vitamins (mL)	1	1	1
BA (mg/L)	0.25	0.25	–
NAA (mg/L)	–	–	0.5
Sucrose (g/L)	30	30	30
Technical agar (g/L)	8	8	8
pH	5.7	5.7	5.7
P.P.F.D. ( $\mu\text{mol}/\text{m}^2/\text{s}$ )	35	35	35

chamber conditions as reported above. Calculate the shoot multiplication rate after 1 month (see Note 4).

3. Singularize the shoots, 2.5 cm long, by a transverse cut, discarding the explants base of the clusters coming from the multiplication media; transfer the shoots onto the rooting medium (Table 1). Set the growth chamber conditions as reported in step 1. After 1 month, calculate the rooting percentages and the average number of roots per shoot (see Note 5).

### **3.5. Aqueous K-IBA Solution for In Vivo Rooting**

1. Aqueous K-indole-3-butyric acid (K-IBA) solutions: weigh 500 or 1,000 mg K-IBA, dissolve in 23–30 mL distilled water in a volumetric flask by gentle stirring until completely dissolved, and raise the volume to 100 mL with distilled water at room temperature.
2. Singularize the shoots, discard the explants base (2 mm), dip the base of the explants in the aqueous K-IBA solutions (5,000 or 10,000 ppm) for 30 s.
3. Immediately transfer the dipped explants to the acclimatization glasshouse in plastic alveolar pots under mist system, 10 s vaporization every 30 min, with 70% relative humidity (see Note 6).

### **3.6. In Vitro Shoot Regeneration from Leaf Fragments**

Cut leaves from in vitro-grown shoots, about  $0.5 \times 0.5$  cm, place them with the lower leaf surface in contact with the media in Petri dishes (see Subheading 3.1, step 9); store in darkness at  $21 \pm 1^\circ\text{C}$  for 4 weeks. Carefully remove the regenerated shoots (see Note 7).

### 3.7. Acclimatization

1. Transfer the rooted and also non-rooted shoots, 3.5-cm high, in alveolar pots prepared as reported in Subheading 2.4, place for 21 days in the glasshouse with 70% relative humidity, maintained by mist system (10 s every 30 min), and then decrease the humidity up to 60% (see Note 8).
2. The mean temperature can vary between 20 and 25°C and, for lighting, use natural light with 50% shade provided by polyester-aluminum net.
3. After 3 months transfer the acclimatized plants to 11-cm diameter pots filled with the same substrate.

## 4. Notes

1. Five species were screened for their sterilization efficiency during different collection time, i.e., *H. heteromalla* “Snow Cap” (SC), *H. petiolaris* (P), *Hydrangea involucrata* “Yoraku Tama” (YT), *H. macrophylla* “Lemon Wave” (LW), and *H. quercifolia* “Snow Queen” (SQ). Genotype SQ gave high percentage in October (87.5%), thus no other explants were sterilized in the following season; LW gave also sterilized explants in autumn, but gave the best results in May (100%); SC and P sterilized explants were obtained with good percentage only in April and May (100% and 83%, respectively); YT gave a low percentage of sterilization only in May (25%) (Table 2).

**Table 2**

**Explants sterilization: different response related to different species and the month of collection**

	Abbreviation	Autumn	Spring	
		October (% of aseptic explants)	April (% of aseptic explants)	May (% of aseptic explants)
<i>H. heteromalla</i> “Snow Cap”	SC	0	25	100
<i>H. petiolaris</i>	P	0	33	83
<i>H. involucrata</i> “Yoraku Tama”	YT	0	0	25
<i>H. macrophylla</i> “Lemon Wave”	LW	16.7	25	100
<i>H. quercifolia</i> “Snow Queen”	SQ	87.5	–	–

**Table 3**

**Sterilization percentages, average of four *Hydrangea* species (SC, P, YT, and LW) related to the concentration of active chlorine applied for 15 min; all explants were treated first with 70% ethanol for 30 s and after chlorine treatment they were washed twice in sterile distilled water**

	Active chlorine content (% w/v)		
	1.5	2.5	5
Sterilization (%)	4.2 ± 4.2	11.2 ± 3.7	77 ± 17.8

Observations made 15 days after treatments

- In order to evaluate the best condition for explants sterilization, three active chlorine concentrations were compared calculating a general average among the four species: SC, P, YT, and LW (Table 3). All the sterile explants, obtained after treatment with 5% active chlorine, were viable and immediately developed new shoots. High concentrations of sterilizing agents were also used in *H. macrophylla* (10) and the results showed 100% sterilized explants with the combination of 2.5% active chlorine and 0.2–0.4% mercuric chloride. However, mercuric chloride should be avoided owing to high toxicity.
- Experiments were carried out for comparison of the multiplication media, containing BA (0.25 or 0.5 or 1 mg/L) or Kin (1 or 2 mg/L) (9). In respect to the control, BA induced the highest multiplication rate of genotype SQ (up to 7 shoots per explants per month) and the highest cluster fresh weight (Fig. 1). The increase of shoot hyperhydration was observed at increasing concentration of BA. However, the use of Kin guaranteed high-quality explants, but the multiplication rate was poor, as the best value reached (2.5) was insufficient for commercial production. The 0.25 mg/L concentration of BA (Table 1) is a good compromise between multiplication production rate (6.5) and shoot quality (9). *H. macrophylla* “Nachtigall” can be multiplied in the presence of 0.2 mg/L BA and 1 mg/L gibberellic acid ( $GA_3$ ), Gamborg-B5 vitamins (15), 10 g/L sucrose, and 15 g/L glucose. On this modified medium, the multiplication rate is 5.2 in permanent liquid culture and only 1.2 on solidified medium (11).
- H. macrophylla* “Nachtigall” grown in TIS showed very good shoot quality without any callus formation and any tissue hyperhydration; the multiplication rate could be enhanced by

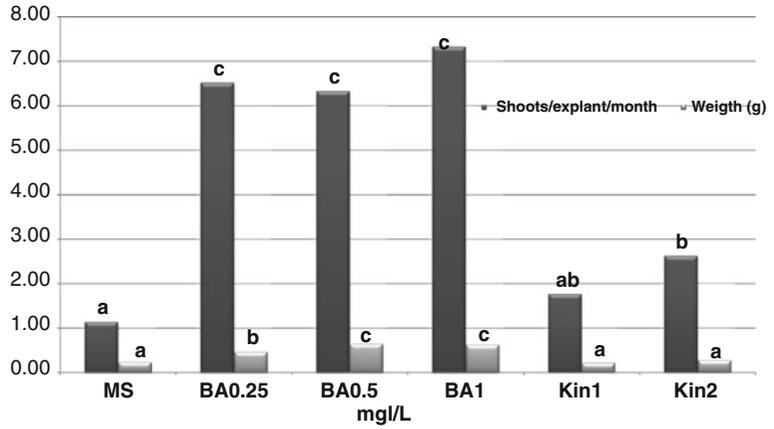


Fig. 1. *Hydrangea quercifolia* “Snow Queen” multiplication rate and fresh weight related to the cytokinin tested (9). Different letters indicate average values which differ at  $p \leq 0.05$  by the SNK test.

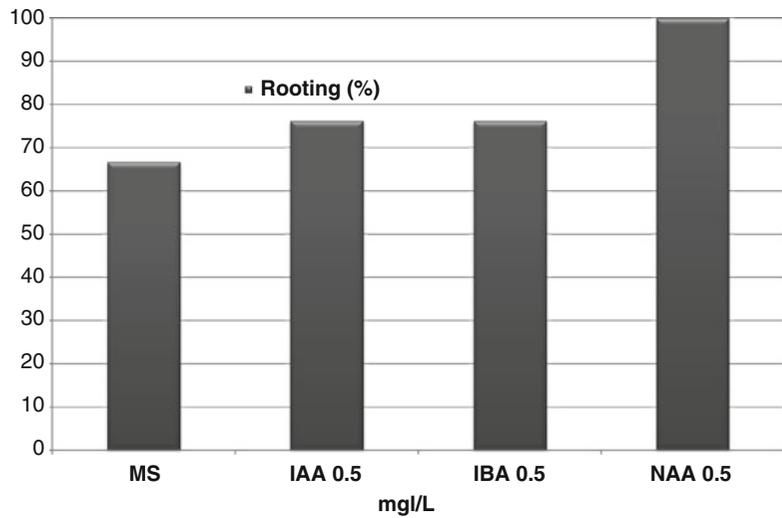


Fig. 2. In vitro multiplication phase: *H. quercifolia* “Snow Queen” explant cultured on MS medium plus 0.25 mg/L BA.

increasing the 8 flooding/day (11). *H. quercifolia* “Snow Queen” grew well in TIS (Fig. 7), showing a higher weight and multiplication rate, over 9 shoots per explant per month. Moreover after 30 days, root primordial appeared at the explant base and the plantlets could be promptly acclimatized (100%).

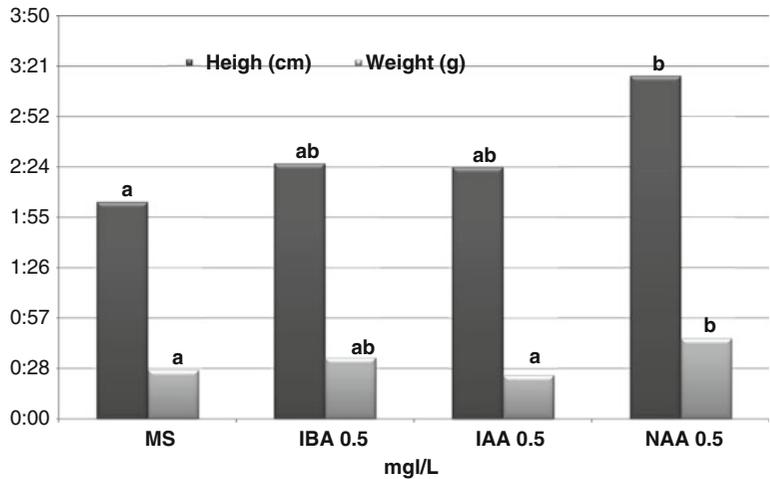


Fig. 3. *H. quercifolia* “Snow Queen” explants in Temporary Immersion System (TIS), after 30 days of culture under a cycle of 3 min flooding every 3 h. Different letters indicate average values which differ at  $p \leq 0.05$  by the SNK test.

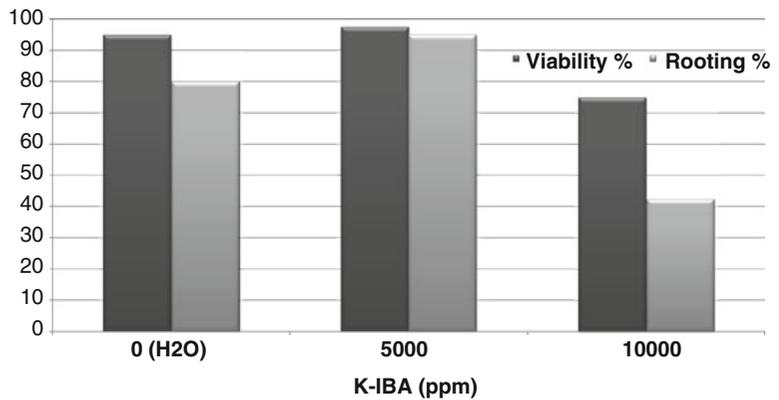


Fig. 4. In vivo rooting percentage of *H. quercifolia* “Snow Queen,” in relation to different auxins at the same concentration (9).

5. For rooting, several media, supplemented with 0.5 mg/L of IBA or NAA or IAA, were tested in comparison with the hormone-free control (9). The rooting percentage of *H. quercifolia* “Snow Queen” reached 60% without plant growth regulators. Among auxins, the highest in vitro rooting rate was achieved in the presence of 0.5 mg/L NAA (100% rooting, Fig. 2) that also induced 8 roots per shoot per month, with large number of thin secondary roots (Fig. 5) and callus



Fig. 5. *H. quercifolia* “Snow Queen” rooted plantlets by treatment with 0.5 mg/L 1-naphthalenacetic acid (NAA).

formation; NAA induced the highest shoot weight and height, too (Fig. 6). In the presence of IBA and IAA the rooting percentage reached 75% (Fig. 2), but the explant base showed lower callus formation than NAA rooted explants.

6. For in vivo rooting, SQ micro shoots, dipped in K-IBA solution at 5,000 or 10,000 ppm concentrations, started rooting after 21 days, showing an excellent viability (up to 98%). The root development was observed, in particular, with 5,000 ppm K-IBA (95%, Fig. 4). The highest K-IBA concentration (10,000 ppm) inhibited both viability and rooting. The plantlets treated with 5,000 ppm K-IBA showed a very good behavior and quality; in this case rooting and acclimatization occurred together, saving propagation time.
7. Experiments were done testing the plant growth regulator contents reported in Tables 4 and 5. In *H. quercifolia* “Snow Queen” (9), the presence of 0.5 mg/L 2,4-D and 0.5 mg/L Kin, or 2 mg/L 2,4-D alone induced callus formation in 100% leaf fragments (Table 4). With the addition of 1 or 2 mg/L BA, shoots regenerated directly, without any intermediate callus formation (Table 5 and Fig. 8). The shoots were then transferred to the multiplication medium. In *H. macrophylla* “Hyd1” (13), 100% regeneration was obtained from leaf



Fig. 6. In vitro rooting of *H. quercifolia* "Snow Queen": evaluation of the height and the fresh weight of rooted shoots (9).



Fig. 7. Proliferation of *H. quercifolia* "Snow Queen" in Temposasy Immersion System.

fragments of in vitro-grown plants using 2.25 mg/L BA and 0.1 mg/L IBA, with 2.2 shoots per explants, on average.

8. In *Hydrangea*, instead of to transfer ex vitro plantlets already rooted, it is possible to maintain a constant high relative humidity (70%) around the aerial part of the shoots to induce

**Table 4**  
**Callus proliferation from leaves grown in vitro on MS media, after 40 days of in vitro cultivation in dark**

PGR content (mg/L)	Fresh weight (g)	Fragments forming callus (%)
None	0.06	0
2,4-D 2 + 2-iP 0.8	0.24	88.9
2,4-D 4 + BA 0.02	0.14	66.7
2,4-D 0.5 + Kin 0.5	0.23	100
NAA 1	0.30	0
NAA 2	0.16	0
NAA 4	0.24	0
2,4-D 1	0.21	66.7
2,4-D 2	0.15	100
2,4-D 4	0.12	88.9

Data are the average of three repetitions (*PGR* plant growth regulator)

**Table 5**  
**Percentage of in vitro leaf fragments showing shoot regeneration related to the used plant growth regulator combination**

PGR content (mg/L)	Fragments showing regeneration (%)
None	0
MS + Zea 0.5	0
MS + Zea 1	11
MS + BA 1	44
MS + BA 2	22

Data recorded after 4 weeks of culture in darkness (*PGR* plant growth regulator)

new roots during the first 15 days of acclimatization. Three-month-old well-acclimatized plants are shown in Fig. 9. The in vitro plantlets with developed roots are faster in acclimatization and in the aerial part development; for commercial purposes it is necessary to evaluate carefully the two options in terms of time consuming and production costs.

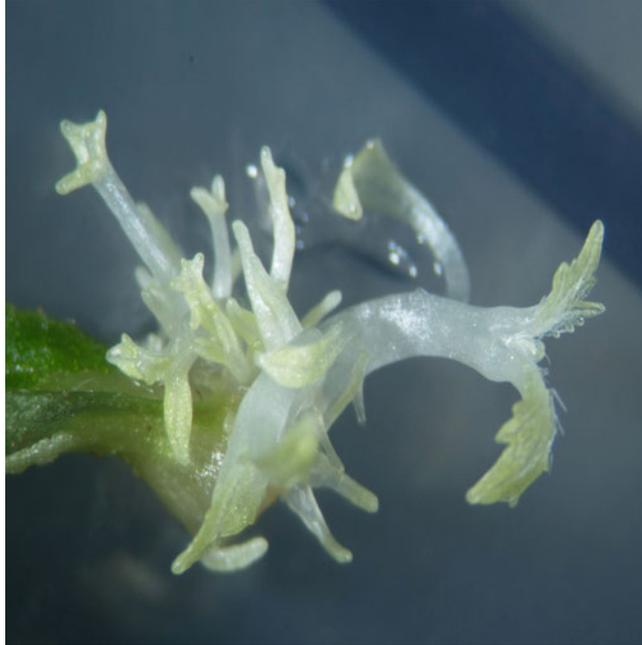


Fig. 8. Regeneration from *H. quercifolia* "Snow Queen" leaves surface, occurring in 2 mg/L BA, after 1 month of culture in darkness at  $21 \pm 1^\circ\text{C}$ .



Fig. 9. Acclimatized *H. quercifolia* "Snow Queen" explants, 3 months after transfer to 11-cm diameter pots, filled with 1:1 peat and sterilized sand (v/v).

## Acknowledgements

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## In Vitro Propagation of Fraser Photinia Using *Azospirillum*-Mediated Root Development

Berta E. Llorente and Ezequiel E. Larraburu

### Abstract

Fraser photinia (*Photinia × fraseri* Dress.) is a woody plant of high ornamental value. The traditional propagation system for photinia is by rooting apical cuttings using highly concentrated auxin treatments. However, photinia micropropagation is an effective alternative to traditional in vivo propagation which is affected by the seasonal supply of cuttings, the long time required to obtain new plants, and the difficulties in rooting some clones. A protocol for in vitro propagation of fraser photinia using the plant growth-promoting ability of some rhizobacteria is described here. Bacterial inoculation is a new tool in micropropagation protocols that improves plant development in in vitro culture. Shoots culture on a medium containing MS macro- and microelements, Gamborg's vitamins (BM), *N*<sup>6</sup>-benzyladenine (BA, 11.1 μM), and gibberellic acid (1.3 μM) produce well-established explants. Proliferation on BM medium supplemented with 4.4 μM BA results in four times the number of shoots per initial shoot that develops monthly. Consequently, there is a continuous supply of plant material since shoot production is independent of season. *Azospirillum brasiliense* inoculation, after 49.2 μM indole-3-butyric acid pulse treatment, stimulates early rooting of photinia shoots and produces significant increase in root fresh and dry weights, root surface area, and shoot fresh and dry weights in comparison with controls. Furthermore, inoculated in vitro photinia plants show anatomical and morphological changes that might lead to better adaptation in ex vitro conditions after transplanting, compared with the control plants.

**Key words:** *Azospirillum brasiliense*, Bacterial inoculation, Fraser photinia, Growth-promoting rhizobacteria, Micropropagation, *Photinia × fraseri*

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### 1. Introduction

Fraser photinia (*Photinia × fraseri* Dress.) is a perennial shrub, belonging to the Rosaceae family, and it is a hybrid between Japanese *Photinia glabra* and Chinese *Photinia serrulata*. It reaches 4–6 m in height and 2–4 m in width. This plant is grown as an ornamental plant in green areas because of striking bright red young leaves which contrast nicely against the dark green mature foliage (Fig. 1a). This shrub has been grown successfully in urban

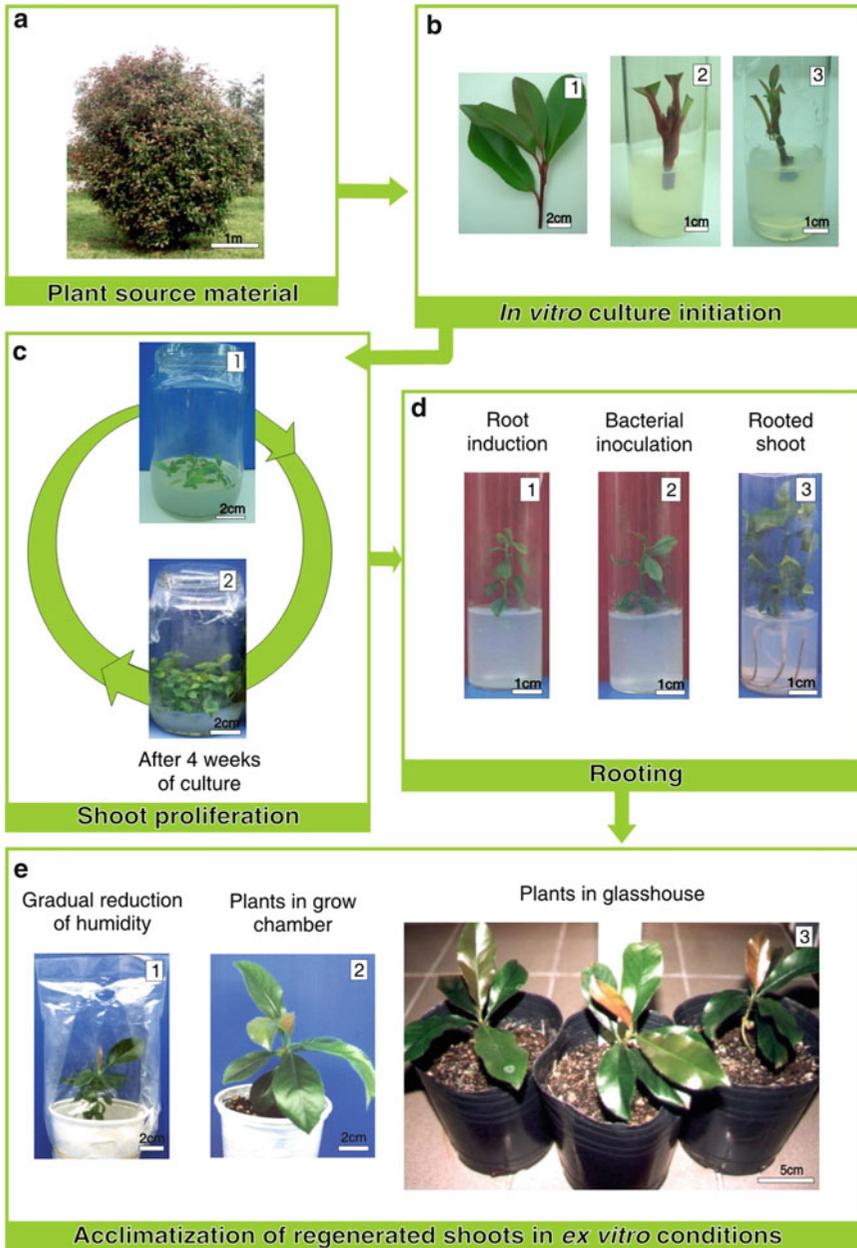


Fig. 1. Stepwise fraser photinia micropropagation. (a) Fraser photinia plant, general aspect. (b<sub>1</sub>) Explant for in vitro culture. (b<sub>2</sub>) Inicial culture in MS supplemented with BA and GA<sub>3</sub>. (b<sub>3</sub>) Established explant. (c) Shoot proliferation in MS supplemented with BA. (c<sub>1</sub>) Inicial. (c<sub>2</sub>) Final. (d) Rooting. (d<sub>1</sub>) Root induction in ½ MS supplemented with IBA. (d<sub>2</sub>) Shoot in ½ MS-hormone free inoculated with *Azospirillum brasilense* Cd. (d<sub>3</sub>) Rooted shoot. (e) Acclimatization in growth chamber. (e<sub>1</sub>) Inicial. (e<sub>2</sub>) Final. (e<sub>3</sub>) Acclimatization in glasshouse. BA: *N*6-benzyladenine. GA<sub>3</sub>: Gibberellic acid. IBA: indole-3-butyric acid. MS: Murashige & Skoog salts supplemented with Gamborg's vitamins.

areas faced with common problems such as air pollution, compact soil, and/or drought. The young red leaves are produced during spring and autumn. The flowering occurs in spring–summer for 2–3 weeks, presenting large, fragrant white terminal clusters (1).

The traditional propagation system of photinia is done by rooting apical cuttings (10–13 cm long) with highly concentrated pulses of

phytohormones (2, 3). The cuttings of this species have shown some difficulties in rooting (4, 5). This fact, together with the seasonal supply of stem cuttings and the long time required to obtain new plants, makes micropropagation the recommended technique for photinia propagation. To our knowledge, the application of *in vitro* techniques for the propagation of this species is very limited (6–10).

In general, it is assumed that micropropagation maintains the fidelity of the donor plant for the sprouting of preexisting apical and lateral primordia. However, *in vitro* culture conditions may produce some biochemical and histological alterations, which modify plant performance, because the structure and function of regenerated plants depend on *in vitro* developmental signals that are related to environmental and nutritional conditions in the culture (11). Therefore, it is very important to find biotechnological methods that avoid any possible alterations.

There are data concerning the effect of microbial inoculants on growth promotion, reduction of hyperhydricity, and other development modifications of *in vitro* cultured plants (9, 10, 12–15). These root-colonizing bacteria exhibit plant growth regulatory activity, induce defense responses in the host, and protect against biotic and abiotic stress, and so they have been defined as plant growth-promoting rhizobacteria (PGPR).

The N<sub>2</sub>-fixing and phytohormone-producing PGPR *Azospirillum brasilense* is Gram-negative, free-living, and highly motile bacteria. It displays a polar flagellum, synthesized during growth in liquid medium, while additional lateral flagella are induced during growth on solidified media. Motility gives the bacterium the ecological advantage of achieving favorable nutrient conditions and optimal oxygen concentrations. It interacts with numerous plant species and positively influences plant growth, N-content, and crop yields. The plant stimulatory effect exerted by *Azospirillum* has been attributed to several mechanisms, including biological nitrogen fixation, production of plant growth-promoting substances, and increase in the rate of mineral uptake by plant roots. The bacterial production of plant growth regulating substances increases the number of lateral roots and root hairs; as a consequence, root adsorption is stimulated (14, 16–19).

It has been reported that *A. brasilense* (strain Cd), in combination with indole-3-butyric acid (IBA, 49.2 µM), applied to shoots as short-time (pulse) treatments, improves *in vitro* rhizogenesis and the quality of micropropagated photinia plantlets (9, 10).

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## 2. Materials

1. Systemic and contact fungicide solution (4% Metalaxil-M + 64% Mancozeb, Fich M™ Cheminova Agro de Argentina S.A.) diluted to 0.2% with tap water.

2. Sodium hypochlorite solution (commercial bleach solution, 5.5 g/L active chlorine).
3. Surfactant Tween 20 solution 0.1% (v/v).
4. Autoclaved distilled water, 150 mL aliquots in 250 mL screw capped bottles.
5. Doubled distilled water.
6. D-Sucrose pure.
7. Agar (e.g., plant cell culture tested Sigma-Aldrich).
8. N<sup>6</sup>-benzyladenine (BA) plant cell culture tested.
9. IBA, plant cell culture tested.
10. Gibberellic acid (GA<sub>3</sub>), plant cell culture tested ≥90% gibberellin A<sub>3</sub>.
11. KOH solutions 0.1, 0.5, and 1.0 M.
12. HCl solutions 0.1, 0.5, and 1.0 M.
13. Basal medium (BM) containing Murashige and Skoog salts (20) and B5 Gamborg's vitamins (21) (Table 1).
14. Okon medium (22) (Table 2).

**Table 1**  
**Plant culture medium**

<b>MS salts (20)</b>	<b>mg/L</b>
KNO <sub>3</sub>	1,900
NH <sub>4</sub> NO <sub>3</sub>	1,650
MgSO <sub>4</sub> ·7H <sub>2</sub> O	370
KH <sub>2</sub> PO <sub>4</sub>	170
CaCl <sub>2</sub> ·2H <sub>2</sub> O	440
H <sub>2</sub> BO <sub>3</sub>	6.2
MnSO <sub>4</sub> ·H <sub>2</sub> O	16.9
ZnSO <sub>4</sub> ·7H <sub>2</sub> O	8.6
Na <sub>2</sub> MoO <sub>4</sub> ·2H <sub>2</sub> O	0.25
CuSO <sub>4</sub> ·5H <sub>2</sub> O	0.025
CoCl <sub>2</sub> ·6H <sub>2</sub> O	0.025
KI	0.83
FeSO <sub>4</sub> ·7H <sub>2</sub> O	27.8
Na <sub>2</sub> EDTA·2H <sub>2</sub> O	37.3
Sucrose	30,000
<b>Gamborg's B5 vitamins (21)</b>	
Thiamine HCl	10
Pyridoxine HCl	1
Nicotinic acid	1
Myo-inositol	100
pH	5.8

**Table 2**  
***Azospirillum* culture media**

	Okon medium (22) (mg/L)	RC medium (23) (mg/L)
K <sub>2</sub> HPO <sub>4</sub>	6,000	500
KH <sub>2</sub> PO <sub>4</sub>	4,000	–
MgSO <sub>4</sub> ·7H <sub>2</sub> O	200	200
NaCl	100	100
CaCl <sub>2</sub> ·2H <sub>2</sub> O	20	20
NH <sub>4</sub> Cl	1,000	–
FeEDTA	65	–
FeCl <sub>3</sub> ·6H <sub>2</sub> O	–	15
Malic acid	5,000	5,000
KOH	5,000	4,800
Yeast extract	50	500
Na <sub>2</sub> MoO <sub>4</sub> ·2H <sub>2</sub> O	2	–
MnSO <sub>4</sub> ·H <sub>2</sub> O	2.35	–
H <sub>3</sub> BO <sub>3</sub>	2.8	–
CuSO <sub>4</sub> ·5H <sub>2</sub> O	0.08	–
ZnSO <sub>4</sub> ·7H <sub>2</sub> O	0.24	–
Congo red	–	40
Agar	–	20,000
pH	6.8–7	6.8–7

15. RC medium (23) (Table 2).
16. Potting media consisting of soil (A horizon from a typical Argiudol), peat (commercial turf Terrafertil™), and perlite in a 5:1:1 by volume.
17. Screw cap tubes.
18. Sterile Petri dishes (9 cm diameter).
19. Glasswares: Erlenmeyer flask, 250 mL, beaker, 1,000 mL, glass flasks, 350 mL (6.4 × 11 cm), flat-bottom glass tubes, 55 mL capacity (2.7 × 10 cm) or 45 mL capacity (2.4 × 10 cm).
20. Scalpels, forceps, spirit burner to flame sterilize instruments.
21. Loop.
22. Pruning shears.
23. Plastic pots (8 and 12 cm diameter).

24. Laboratory transparent film (30–150  $\mu\text{m}$  thick).
25. Transparent plastic bags.
26. Micropipettes, 100 and 1,000  $\mu\text{L}$ .
27. Sterile tips.
28. Incubator 20–60°C.
29. Orbital shaker.
30. Magnetic stirrer and magnetic bar.
31. Microwave cooker.
32. Laminar flow bench.
33. Culture room or growth chamber.
34. Glasshouse.

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### 3. Methods

This protocol describes micropropagation of photinia via axillary branching, employing both auxin induction pulses on culture media and inoculation with *A. brasilense* to improve rooting.

#### **3.1. Preparation and Sterilization of Plant Culture Media**

1. Prepare the BM supplemented with 3% (w/v) sucrose in doubled distilled water. Store at 4°C.
2. Prepare stock IBA and GA<sub>3</sub> solutions by dissolving the powder in a few drops of KOH 0.1 M and then adding doubled distilled water to make up the volume to 1.0 mM. Store at –20°C.
3. Prepare stock BA solution dissolving the powder in a few drops of HCl 0.1 M. Add doubled distilled water to make up the volume to 1.0 mM. Store at –20°C.
4. Supplement BM with 11.1  $\mu\text{M}$  BA and 1.3  $\mu\text{M}$  GA<sub>3</sub> from stock solutions (initiation medium).
5. Supplement BM with 4.4  $\mu\text{M}$  BA (proliferation medium).
6. BM is modified to serve as a rooting medium (RM) by reducing the concentration of MS mineral salts to half the usual strength, and of sucrose to 2% (w/v).
7. For rooting induction transfer to RM containing 49.2  $\mu\text{M}$  IBA, and for root development use RM without IBA.
8. Adjust the pH of the media to 5.8 with HCl or KOH (1.0, 0.5 or 0.1 M).
9. Add agar 0.7% (w/v) for the initiation and proliferation media and 0.6% (w/v) for rooting media. Dissolve by microwaving.
10. Dispense the media into suitable containers, 10 or 15 mL aliquots into 45 or 55 mL capacity flat-bottom glass tubes for the

shoot initiation and rooting medium, and 60 mL aliquots into 300 mL glass bottles for shoot proliferation.

11. Sterilize the media by autoclaving at 121°C for 20 min (101 kPa steam pressure).
12. Store the autoclaved media in the dark at room temperature for a maximum of 20 days.

### **3.2. Surface Sterilization of Plant Source Material**

1. Select vigorous, disease-free stock plants grown under field conditions (Fig. 1a).
2. Spray the parental stock plants with fungicidal solution 48 h before initiating tissue culture (see Note 1).
3. Cut shoot tips (40–60 mm) of actively growing shoots from semi-hardwood stems of adult photinia plants (Fig. 1b<sub>1</sub>; see Note 2).
4. Remove leaf blades (see Note 3).
5. Pre-disinfect the sections with 2 g/L fungicide (Fich M™) for 60 min, then wash them for 2 h under running water. It is recommended that the protocol runs separately for each clone (see Note 4).
6. Disinfect the surface of explant sections by immersion in 20% sodium hypochlorite solution, containing 2 drops of surfactant in a sterile beaker, and stirring on a magnetic stirrer for 30 min (see Note 5).
7. Sterilize the laminar flow surface before use.
8. Rinse in sterile distilled water three times to remove the disinfectant and store there until culture initiation (no longer than 1 h).

### **3.3. Initiation of Culture**

1. Sterilize and flame all instruments and laminar flow surface.
2. Working on a laminar flow bench, remove the base of each axenic explant damaged by the disinfectant, and place in a sterile Petri dish. The use of at least 30 explants per genotype is recommended.
3. Insert the base of single-node explants into the surface of the initiation medium in a flat bottom tube. Make sure that the base of the shoot is inserted firmly in the medium (Fig. 1b<sub>2</sub>).
4. Seal the tube with laboratory transparent film.
5. Incubate in room or growth chamber at 24 ± 2°C under Phillips fluorescent daylight tubes (30–40 μmol/m<sup>2</sup>s) with a 16 h photoperiod.
6. Recommended parameters to evaluate: number of leaves, shoot length, frequency of shoot sprouting, number of regenerated shoots per explant, presence of callus, and the number of heterohydric plantlets for each genotype.
7. Transfer elongated shoots (4–6 weeks) to the proliferation medium (Fig. 1b<sub>3</sub>).

**3.4. Shoot Proliferation**

1. Sterilize and flame all instruments and laminar flow before use.
2. In laminar flow, eliminate hyperhydric, malformed, oxidized, and dead tissues (see Note 6). Also, remove callus (see Note 7) and cut the basal part of regenerated shoots.
3. Cut main and lateral shoots into nodal segments of a similar size (~20 mm) and transfer to 350 mL flasks (8–10 shoots per flask) with 60 mL sterile proliferation medium (Fig. 1c<sub>1</sub>).
4. Seal the tube with laboratory transparent film.
5. Incubate and subculture the shoots at 4-week intervals in the same fresh medium for continued proliferation (Fig. 1c<sub>2</sub>).
6. Recommended parameters to evaluate: leaf number, length of main and lateral shoots, lateral sprouts per shoot, proliferation rate (relationship between final and initial nodes numbers), percentage of hyperhydric shoots, and callus growth (see Note 8).

**3.5. Bacterial Culture**

1. Use Cd (ATCC 29710) *A. brasilense* strain.
2. Maintain the strain on RC medium (23) slope in the dark at 4°C, and subculture at regular 3-month interval (see Note 9).
3. Remove loopfuls of *A. brasilense* bacterial colonies from the stock culture and inoculate 150 mL medium (22) with *A. brasilense* in 250 mL sterile Erlenmeyer flask.
4. Incubate at 34 ± 2°C on an orbital Sontec™ shaker (140 rpm) for 72 h (see Note 10).

**3.6. Rooting Induction**

1. Sterilize and flame all instruments and laminar flow before use.
2. Select elongated shoots (~30 mm long) with 2–4 nodes, coming from 4 to 5 weeks of culture on the proliferation medium.
3. Remove hyperhydric, malformed, oxidized, and dead tissues and basal callus under laminar flow.
4. Insert the base of shoots into 20 mL aliquots of rooting induction medium containing 49.2 μM IBA.
5. Seal the tube with laboratory transparent film (Fig. 1d<sub>1</sub>).
6. Incubate the cultures for 6 days for root induction pulse treatment.

**3.7. Inoculation of Root-Induced Shoots and In Vitro Development**

1. Subculture the photinia shoots in root development medium (growth regulator-free) in a flat bottom tube with 20 mL aliquots of RM medium (see Note 11).
2. At the time of transferring induced shoots to hormone-free medium, add 0.1 mL bacterial culture (initiated 72 h before) at the base of each explant (Fig. 1d<sub>2</sub>). Use treatments without inoculation as control (see Note 12).

3. Seal the tube with laboratory transparent film.
4. The shoots are considered as rooted if they possess at least one 10 mm root after 40 days of culture (Figs. 1d<sub>3</sub> and 2a, b).
5. Recommended parameters to evaluate: fresh and dry weights of shoots and roots, percentage of rooted shoots, emerging time of the first root, main root length, number of roots per shoot and root surface area (see Notes 13 and 14). In addition, anatomical studies provide valuable information on *in vitro* development (see Notes 15 and 16).
6. Survival rate *A. brasilense*, strain Cd, on the root surface, bacterial concentration, and eventual contamination, could be evaluated by culturing inoculated roots in *Azospirillum* liquid medium (22) and checking viable cells by dilution plate counts on RC medium (23) supplemented with 2% agar.

### **3.8. Acclimatization of Regenerated Shoots in Ex Vitro Conditions**

1. Remove each rooted shoot carefully from the culture medium, eliminate as much agar as possible without injuring the root structure and wash the roots thoroughly with tap water to remove any trace of the medium.
2. Transfer plants individually to 8 cm diameter plastic pots, each containing the acclimatization mixture (see Subheading 2, item 16).
3. Irrigate the plants thoroughly.
4. Enclose the plants in their pots in transparent plastic bags (Fig. 1e<sub>1</sub>). Maintain the regenerated plants in acclimatization conditions. At the initial acclimatization stage, incubate the regenerated *in vitro* plants in a growth room at 24 ± 2°C with a 16 h photoperiod and 40–60 μmol/m<sup>2</sup>s light intensity.
5. After 7 days, remove a corner from the top of each bag to reduce the relative humidity and to facilitate acclimatization of the plants in *ex vitro* conditions. Glasshouse conditions oscillate (night to day) from 0 to 300 μmol/m<sup>2</sup>s light intensities, 20–40°C temperature and 95–40% relative humidity.
6. Gradually remove the remaining top corner of the bags after a further 2 weeks and discard the bags after an additional week (Fig. 1e<sub>2</sub>; see Note 17).
7. Transfer the plants to 12 cm diameter plastic pots in the glasshouse.
8. After 4–6 weeks in the glasshouse, plants developed stems with secondary thickening, opposite elliptic leaves with serrated margin, and roots well developed. Expanded leaves have glossy green color (Fig. 1e<sub>3</sub>).
9. Suggested parameters to evaluate: survival, rates of proliferation, and weekly elongation.

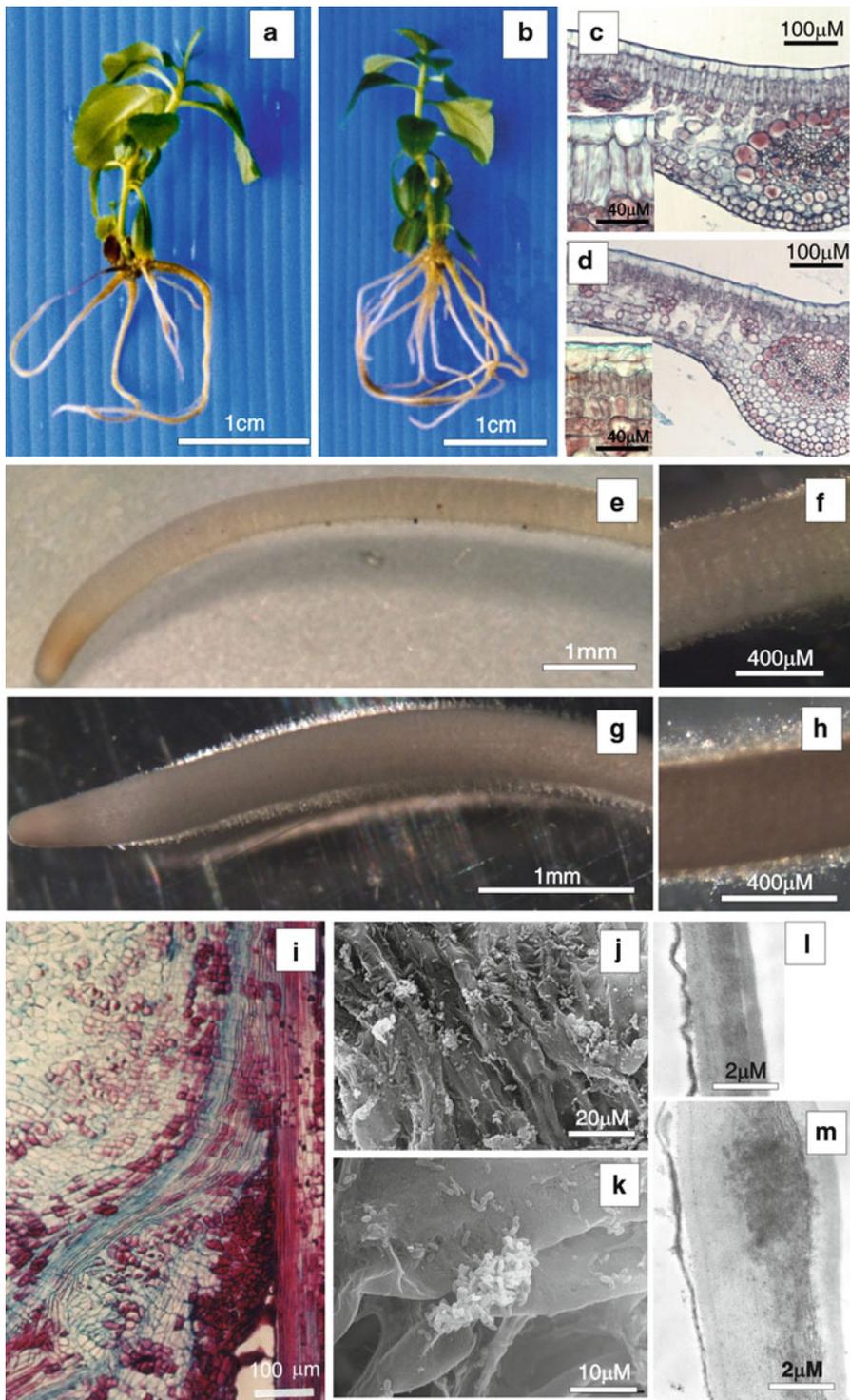


Fig. 2. Anatomical and morphological changes of in vitro fraser photinia plants improved by *Azospirillum brasilense*, strain Cd. (a, c, e, f, l) Non-inoculated plants; (b, d, g, h, i, j, k, m) inoculated plants. (a, b) General aspect of in vitro plant. (c, d) Leaf transverse section by light microscopy (inset: palisade parenchyma detail). (e–h) Roots of in vitro rooted plants by stereoscopic microscopy. (e, g) General view of root apical and root hair zone. (f, h) Hair zone detail. (i) Vascular connection between root and shoot, longitudinal section by light microscopy. (j, k) Aggregated bacteria on root hair surface by scanning electron microscopy. (l, m) Cuticle and outermost wall of leaf epidermal cell by transmission electron microscopy.

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## 4. Notes

1. Fungal and bacterial contamination is often a major difficulty in establishing photinia *in vitro* cultures similar to other ornamental foliage plants (24). Mildew, fire blight, and leaf spot diseases cause serious problems in this species (1). The fungicidal solution treatment of stock plant, used to initiating tissue culture, reduces the loss of explants by contamination.
2. It is possible to culture meristems instead of stem nodal sections. This implies isolation of apical dome and 1–2 leaf primordia under the binocular microscope. The procedure is more laborious but has the advantage of producing virus-free plants (25).
3. Shoot branching is controlled by growth and development of buds in the axils of leaves. Axillary buds are indeterminate structures that may be in a state of dormancy; however, under endogenous or environmental stimuli, they can switch to sustained growth. A young leaf produces a large amount of auxin that flows out through the petiole inhibiting activity in the abscission zone. If the leaf blade is damaged, leaf auxin synthesis decreases and bud outgrowth is enhanced by modification of auxin/cytokinin rate (26).
4. Generally, each plant has a different microbial content. Therefore, the combination of different plants can increase loss by cross contamination. In addition, individual management permits the study of *in vitro* responses of each clone due to the inherent genotypic-specific differences and endogenous content of phytohormones. Although the *in vitro* responses of different genotypes of photinia have not been described, variable reactions have been detected in several species (25).
5. The success rate for disinfection depends on the health status of the source plant and the time of the year in which the *in vitro* establishment is performed. More than 60% explants, cut in spring and summer, are obtained without contamination following the disinfection method explained above (9).
6. Explants in *in vitro* environmental and chemical conditions may generate hyperhydric (or “vitrified”) organs with a water-soaked appearance. They are fragile, show an abnormal structure and survive very poorly (11).
7. The development of callus at the shoot base may produce adventitious shoots showing somaclonal variation. This genetic/epigenetic instability can be employed to generate variants of a commercial cultivar; however, when clonal genetic fidelity is required it should be avoided (25). Photinia shoots cultivated by the described methodology show incipient basal

- callus in 10% shoots but has no effect on shoot development (9). The initiation of nodal explants on Woody Plant Medium (WPM) (27) supplemented with 2  $\mu\text{M}$  BA produced large calli that usually developed from the basal end of each explant (6).
8. The addition of BA in MS medium increases the lateral sprouts; proliferation rate reaches up to 4.3 in the presence of 4.4  $\mu\text{M}$  BA. The stock retains shoot proliferation capacity for at least 24 months (9). However, other researchers recommend optimal BA concentration 44.4  $\mu\text{M}$  (8), 8  $\mu\text{M}$  (6) or 2.2  $\mu\text{M}$  (7) in the culture media for shoot induction of photinia. Earlier the shoot proliferation rate was obtained 3.7 after 56 days of culture (7) and the highest usable shoots (4.3) were produced after 35 days in WPM medium (6).
  9. Colonies that develop in Petri dishes of RC medium incubated at 37°C for 96 h showed characteristics: scarlet color, abundant growth, dry consistency, 1.5–2 mm diameter, round or irregular form, undulated edge, and rugose colony type with ridges radiating from the center. The scarlet coloration readily distinguishes *Azospirillum* spp. from colonies of other diazotrophs. Microscopic examination reveals rods with fat droplets and active characteristic movements of *Azospirillum* spp. (23).
  10. Bacterial cell density  $10^6$ – $10^8$  c.f.u./mL permits the growth of both shoots and bacteria. By using bacteria-sterile culture medium, no statistical differences were observed as compared to control in the evaluated parameters (9).
  11. Continuous culture of photinia microshoots in the media containing 0.5–25  $\mu\text{M}$  auxins did not stimulate in vitro rooting. Higher concentrations of IBA (49.2, 147.6, 295.3, or 442.9  $\mu\text{M}$ ) and exposure time of microshoots (1, 3, 6, or 15 days) to IBA (pulse treatments) showed 60–100% rooting without indicating any statistical analysis differences (8).
  12. Although root development occurs also in hormone-free medium after auxin pulse treatment, the *Azospirillum*-inoculated plants show enhancement in shoot and root growth parameters (9, 10) (Fig. 2a, b; see Notes 14 and 15).
  13. Root surface area from in vitro plants is determined by immersing air-dried roots in  $\text{Ca}(\text{NO}_3)_2$  saturated solution and removal of amount of salt (mg) from the solution by adhesion to the root surface (28).
  14. Photinia shoots growing on medium amended with 49.2  $\mu\text{M}$  IBA for 6 days and inoculated with *A. brasilense* significantly increase fresh and dry weights of the shoots (32% and 62%, respectively), fresh and dry weights of roots (105% and 137%, respectively), root surface area (65%), and rooting percentage as compared with the control (9).

15. Anatomical and morphological studies show that micropropagated photinia inoculated with *A. brasilense* develops better than when subjected to auxin treatment as a control; it shows leaves with two layers of palisade parenchyma (Fig. 2c, d), a thick cuticle (Fig. 2l, m), and linear unicellular trichomes. There is no proliferation of undifferentiated tissue and plants show shoot–root vascular connection (Fig. 2i). The stomata distribution in *in vitro* inoculated plants is similar to that in *ex vitro* leaves. In addition, plants treated with *A. brasilense* have a large root hair zone over the root surface (10) (Fig. 2e–h).
16. *A. brasilense*, strain Cd, forms aggregates around photinia roots (Fig. 2j, k) and sometimes embedded in a mucigel layer on the root surface (10).
17. The *in vitro* culture conditions, high humidity and low levels of light, results in anatomical and physiological changes in micropropagated plants. Humidity should be reduced gradually, so that plants develop normal cuticle and stomata and the survival rate during acclimatization is increased. In the same way, under low light intensity leaves are thin and need to be gradually moved to natural light condition (11, 29).

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## Micropropagation of *Helleborus* through Axillary Budding

Margherita Beruto, Serena Viglione, and Alessandro Bisignano

### Abstract

*Helleborus* genus, belonging to the *Ranunculaceae* family, has 20 species of herbaceous perennial flowering plants. The commercial exploitation of this plant is dependent on the selection and propagation of appropriate lines. High propagation rate could be accomplished by using a suitable tissue culture method enabling the rapid introduction of valuable selections in the market. However, in vitro cultivation of *Helleborus* is still very difficult. Thereby the development of reliable in vitro propagation procedures is crucial for future production systems. Axillary buds cultured on agar-solidified Murashige and Skoog medium supplemented with 1 mg/L benzyladenine, 0.1 mg/L  $\beta$ -naphthoxyacetic acid, and 2 mg/L isopentenyl adenine develop shoots after 16 weeks of culture under 16 h light regime, 50–60  $\mu\text{mol/s/m}^2$ , and  $19 \pm 1^\circ\text{C}$ . The multiplication rate ranges from 1.4 to 2.1. However, the genotype and the number of subcultures affect the efficiency of the micropropagation process. The rooting of shoots is about 80% in solidified MS medium containing 1 mg/L 1-naphthaleneacetic acid and 3 mg/L indole-3-butyric acid. The described protocol provides information which can contribute to the commercial production of *Helleborus* plants.

**Key words:** *Helleborus*, Micropropagation, Axillary bud stimulation, Rooting

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### 1. Introduction

Hellebores are herbaceous perennial plants, belonging to the *Ranunculaceae* family, highly valued for their ornamental qualities. The Christmas rose, *H. niger*, is a well-known representative of this family with winter flowering plants. However, other species, like *H. foetidus* and *H. argutifolius*, as well as crosses of both *Helleborus*  $\times$  *nigercors* and *Helleborus*  $\times$  *hybridus*, are more frequently available in the wholesale trade as pot and cut flower production or distributed in ornamental gardens. Economic data from Dutch auctions show that *Helleborus* has gained increasing importance over the years. According to VBN data (Vereniging van Bloemenveiligen in Nederland—Association of Flower Auctions, the Netherlands), during the period 2007–2009 about 1.5 millions cut flowers of

*Helleborus* and 500 thousand pot plants of *Helleborus* (average unit price: 0.46–0.52 /cut flower stem; 3.46–3.78 /pot plant) were sold in the Netherlands. Therefore, the choice of a proper selection line and its propagation are key factors for commercial exploitation of this plant. *Helleborus* plants are reproduced by both generative (seed) and vegetative (in vivo division of rhizomes and micropropagation) propagation. The in vivo propagation methods are generally time-consuming and therefore the best-quality hellebore plants are expensive to buy. Seed propagation system facilitates to have a relatively large number of individuals, but a certain amount of variation can occur. On the other hand, in vivo division ensures that all the offsprings are identical; however this is a very slow propagation approach. Tissue culture methods have successfully been used for high propagation rates of ornamentals, resulting in the rapid introduction in the market of valuable selections. However, in vitro cultivation of *Helleborus* is not yet free of difficulties, as the success of micropropagation is highly dependent on the species and clone. So far, no comprehensive protocol for *Helleborus* micropropagation has been published, even though micropropagated plant material is available in the market. A few published reports on in vitro propagation of *Helleborus* lack of detailed information about cultivation methods and prospects of commercialization (1, 2). Moreover, the root development and the subsequent in vivo establishment of microplantlets would require more refinement to produce reliable results and allow for commercial exploitation.

For *H. orientalis*, Lim and Kitto proposed the ex vitro rooting of microshoots with a rather low survival percentage (50–56%) (3). Controversial results in the use of auxins to stimulate root development in *H. niger* were reported by Seyring (4) and Poupet et al. (5). Dhooghe and Van Labeke carried out the ex vitro rooting process for four *Helleborus* species which were pretreated at 5°C with IBA and NAA at 5°C over a week period, but no data on rooting percentage and survival efficiency was provided (6). It has also been reported that the phase of in vitro multiplication can markedly affect the further rooting efficiency of *Helleborus* plantlets (7).

This chapter describes a detailed protocol for in vitro propagation of *Helleborus* × *nigercors* genotypes, selected for their commercial potential for the Mediterranean area, both as cut flowers and pot plants.

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## 2. Materials

1. Adult plants of *Helleborus* × *nigercors* are cultivated according to the traditional growing practices (8). In vitro cultures are initiated from axillary buds collected in spring time.

2. Tissue culture facilities: laminar-flow cabinet, autoclave, culture room, magnetic stirrer, refrigerator, glass bead sterilizer, forceps, scalpels, pipettes, culture test tubes (25 × 150 mm) closed by a plastic cap, and culture vessels (Ø 7.5 cm, 320 mL) closed with polycarbonate lids and sealed by a polyethylene film.
3. Surface sterilization solutions: 70% ethanol (v/v), 0.5% mercuric chloride (w/v), sodium hypochlorite (commercial bleach, 13–14% v/v), and doubled distilled water (conductivity,  $2 \pm 0.5 \mu\text{S}/\text{cm}$ ).
4. Solidified media supplemented with a modified formulation of Murashige and Skoog (MS; (9)): include all chemicals, vitamins, and plant growth regulators as mentioned in Table 1.
5. PPM, Plant Preservative Mixture (Plant Cell Technology Inc™, USA).
6. Polyethylene film (Domopack™, USA).
7. Acclimatization facilities: tap water, pots (Ø 10 cm), polypropylene film (17 g/m<sup>2</sup>), green shading net (70%), iprodione (Rovral®), fertilizers (NPK—14:16:18 at stage 0, and NPK+Mg—19:6:30+3 at stage 4).

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### 3. Methods

#### 3.1. Preparation and Sterilization of Culture Media

Two different media are used: M1 for initiation and multiplication phase and M2 for rooting phase (Table 1). To prepare 1 L of culture medium, add all ingredients according to Table 1. The pH of all media is adjusted at  $5.8 \pm 0.1$  while the media are in sol-status ( $T = 75 \pm 1^\circ\text{C}$ ). The media are poured into tubes (10 mL; initiation phase) and vessels (100 mL; multiplication and rooting phase) and autoclaved at  $120^\circ\text{C}$ , 101 KPa, 15 min.

#### 3.2. Preparation of Donor Plants (Stage 0)

Although *Helleborus* is a tolerant plant and so far no important pest and diseases are reported, it is mandatory to initiate in vitro propagation from mother plants that went through phytosanitary controls. Mother plants are grown with standard nursery practices (8). Two months before the initiation of cultures, transfer rhizomes with freshly adventitious roots to pots (Ø 18 cm) filled with clay. Care should be taken that the newly formed buds remain on top of soil (Fig. 1a).

#### 3.3. Initiation of Culture (Stage 1)

1. Axillary buds, about 1–2 cm long, are taken from mother plants (Fig. 1b).
2. Sterilize buds by a quick dip in 70% (v/v) ethanol, followed by 5 min 0.5% HgCl<sub>2</sub> solution (w/v) treatment, and rinse three times with sterile double distilled water. A subsequent NaOCl

**Table 1**  
**Media for micropropagation through axillary bud stimulation of *Helleborus***

Medium code	Initiation culture	Shoot multiplication	Rooting
	M1		M2
Macronutrients (mg/L)			
	Murashige and Skoog (9) mod		
CaCl <sub>2</sub> ·2H <sub>2</sub> O	440		
KH <sub>2</sub> PO <sub>4</sub>	170		
KNO <sub>3</sub>	950		
MgSO <sub>4</sub> ·7H <sub>2</sub> O	185		
NH <sub>4</sub> NO <sub>3</sub>	825		
Microelements (mg/L)			
	McCown Woody Plant Medium (12)		
CuSO <sub>4</sub> ·5H <sub>2</sub> O	0.25		
FeNaEDTA	36.70		
H <sub>3</sub> BO <sub>3</sub>	6.20		
MnSO <sub>4</sub> ·H <sub>2</sub> O	22.30		
Na <sub>2</sub> MoO <sub>4</sub> ·2H <sub>2</sub> O	0.25		
ZnSO <sub>4</sub> ·7H <sub>2</sub> O	8.60		
Vitamins (mg/L)			
	McCown Woody Plant Medium (12) mod		
Glycine	2.0		
Myo-inositol	100.0		
Nicotinic acid	0.5		
Pyridoxine HCl	0.5		
Thiamine HCl	0.4		
Other organic addenda (mg/L)			
Riboflavine	2.5		
Adenine hemisulphate salt	67.0		
Calcium gluconate·H <sub>2</sub> O	500.0		
Sucrose	30,000.0		
Agar-agar	8,000.0		
Plant preservative mixture (Plant Cell Technology, Inc. <sup>TM</sup> , USA) (mL/L)	1.0		
Growth regulators (mg/L)			
2-Naphthoxyacetic acid (NOA)	0.1		–
N6-Benzyladenine (BA)	1.0		–
N6-[2-Isopentyl]adenine (2-IP)	2.0		–
4-[3-Indolyl]butyric acid (IBA)	–		3.0
1-naphthaleneacetic acid (NAA)	–		1.0

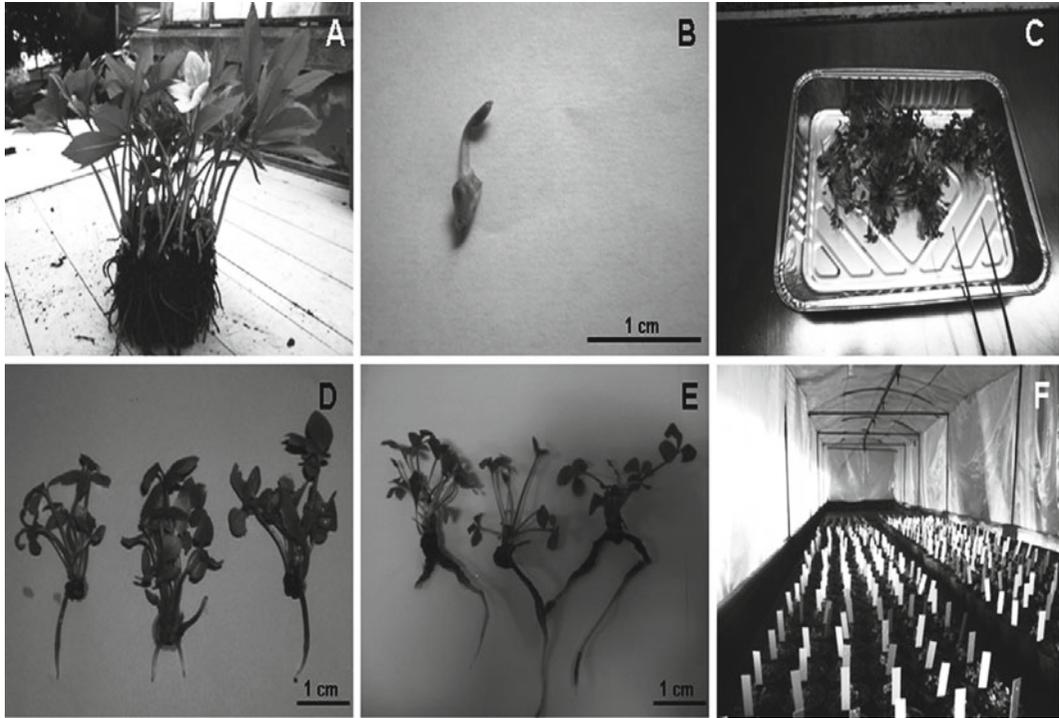


Fig. 1. Different steps in the micropropagation of *Helleborus × nigercors* (coded NI). (a): mother plant; (b): axillary bud used as initial explant; (c): multiplication phase; (d, e): rooted plantlets at the end of rooting phase (4 weeks) performed on gelled M2 medium (d) and gelled M2 medium supplemented with vermiculite (e); (f): acclimatization phase.

(1% available chlorine) treatment is given for 15 min and finally buds are rinsed three times with sterile double distilled water.

3. Remove external leaves from the buds with the help of a sterile scalpel and forceps and transfer buds to a solidified culture medium (M1; Table 1) and incubate under 16 h light/8 h dark, 50–60  $\mu\text{mol/s/m}^2$ , at  $19 \pm 1^\circ\text{C}$  (see Note 1).

### 3.4. Shoot Multiplication (Stage 2)

This phase is based on the growth of axillary buds inoculated in the initiation phase (Fig. 1c). The two first subcultures are made in the same culture tubes as described for the initiation phase; the subsequent subcultures are carried out in culture vessels, 10 shoots per vessel, containing the M1 medium (Table 1). Culture conditions of stage 2 are as described for the initiation phase.

1. After 3–4 weeks from the initiation, transfer explants to fresh M1 (Table 1).
2. After further 12 weeks of culture, developing new axillary shoots are isolated and subcultured on fresh M1 (Table 1). Divide in vitro shoots by transverse section, and carry out subsequent subcultures on the same medium every 6–8 weeks.

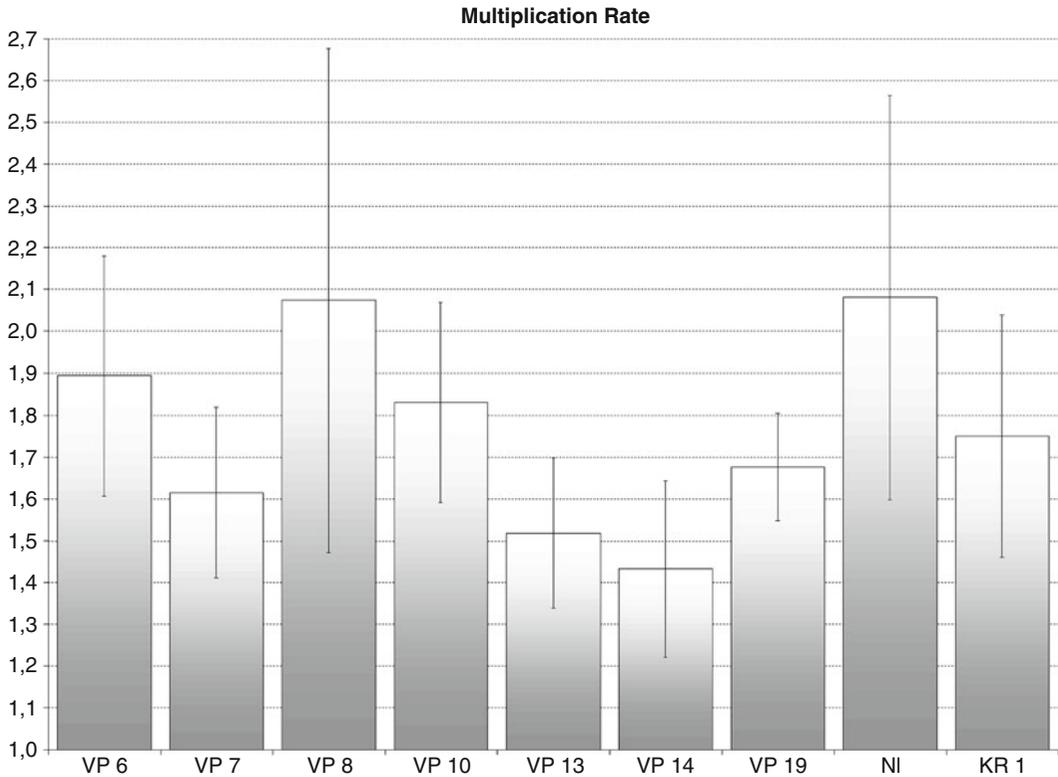


Fig. 2. Average multiplication rate of different *Helleborus × nigercors* genotypes. X-axis, *Helleborus × nigercors* genotypes (coded VP6, VP7, VP8, VP10, VP13, VP14, VP19, NI, and KR1); Y-axis = average multiplication rate based on data collected along the 15 months of culture (three multiplication subcultures).

3. Subsequent subcultures are done over a period of 12–24 months. The multiplication rate varies from 1.4 to 2.1, depending on the genotype (Fig. 2; see Note 2).

### 3.5. Rooting (Stage 3)

A chilling treatment (5–7°C) can enhance the rooting efficiency by 5% (6, 7). However, culture conditions of stage 2, including microelements and macroelements formulation, plant density, concentrations and type of growth regulators, type of gelling agent and light condition, can affect the rooting ability (see Note 3).

1. For rooting, transfer single shoots to solidified rooting M2 medium (Table 1; see Note 4) (10 ml for each tube).
2. After 4 weeks of in vitro rooting, 80% shoots form roots, on average (Fig. 1d, e). However, unrooted shoots display rooting properties when transferred to in vivo conditions; approximately 50% of the unrooted shoots show visible roots after 1 month from the acclimatization.

### 3.6. Acclimatization Phase (Stage 4)

Under the Mediterranean conditions, with mild to cool wet winters and with warm to hot dry summers, it is mandatory to carry out plantlet acclimatization from September to March when the

temperature and the light regimes are optimal to prevent material losses (see Note 5).

1. Transfer shoots with or without visible roots in plastic pots ( $\varnothing$  5.5 cm) containing a substrate of 75% peat and 25% perlite (pH 5.5–6.5). Water the potted plantlets abundantly just after in vivo transplanting, and maintain under unheated greenhouse conditions, protected by a white polypropylene film ( $17 \text{ g/m}^2$ ) and green shading net (70%) over a 2-week period (Fig. 1f).
2. Plants should be maintained at 90–100% humidity level; gradually remove polypropylene film within a month from transplanting and leave the shading net. At this time, give iprodione (Rovral® 1 mL/L) treatment to prevent fungal growth and fertilize plants with a mixture of NPK + Mg–19:6:30 + 3, 0.5 g/L (6).
3. After 2–3 months, plantlets are ready to grow under regular growing condition. The first flowering for the studied genotypes is scored after about 1 year from the ex vitro establishment and the full blooming is recorded within further 2–3 months.

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#### 4. Notes

1. The establishment of axenic cultures of *Helleborus* can be difficult due to micro-organism contaminated source material. This may limit the whole protocol of micropropagation. Although the sterilization protocol described here is quite effective, only 40–50% of healthy shoots are obtained from the cultured explants.
2. *Helleborus* shoots show a different propagation ratio as a function of the genotype. The multiplication rate generally decreases after about 12–15 months of culture. After this period a reestablishment of new shoot lines is necessary. Growth regulators added to the medium can influence the physiological status and the multiplication rate of the shoots. Shoots growing on media supplemented with 0.5, 1, 3, and 5 mg/L BA show lower leaf development, higher multiplication rate, and reduced chlorosis as compared to media containing equimolar concentrations of kinetin.
3. The use of kinetin in the multiplication medium enhances the subsequent rooting efficiency, while benzyladenine (BA) at high concentration (equal or higher than 3.0 mg/L) decreases the rooting percentage (7). Macronutrients used in the multiplication phase can influence the subsequent in vitro rooting: shoots previously multiplied on MS macroelements-based media showed an improved rooting efficiency compared to those

multiplied on Quorin and Lepoivre macroelements (10), once they were transferred to M2 rooting medium (Table 1) (7). The rooting capacity decreases with shoots previously multiplied at high plant density (i.e., higher than 15 shoots per vessel) (7).

4. As an alternative rooting procedure, consider to drench shoots for 1 week in solution of 3.0 mg/L indole-3-butyric acid (IBA) and 1.0 mg/L 1-naphthaleneacetic acid (NAA) at 7°C and transplant to a MS gelled medium with no hormones. This system results in 87% rooting. The rooted shoots transferred under *in vivo* conditions show 85% survival rate (7). Vermiculite-containing gelled media (ratio: 100/80 v/v) improve root development (11), although no significant differences in the rooting percentage are scored when compared with the control (plantlets cultured on rooting solidified medium without vermiculite) (Fig. 1d,e).
5. All hellebores species don't have the same life cycle and similar growing conditions; especially the behavior of hybrids can differ from parents. Light to partial shade is the best for most of species and hybrids. Bright sun tends to burn the leaves, and flowering is poor under the full shade. Species, such as *Corsican hellebore*, are likely to be failure in shade. In autumn, as the trees are shedding their leaves, *H. orientalis* and its hybrids produce a fresh crop of new foliage. Several species are fully deciduous in winter, and *H. vesicarius* and *H. thibetanus* are summer dormant and grow better and flower under sunny springs (8).

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# Chapter 21

## Micropropagation of *Cordyline terminalis*

Tui Ray, Prasenjit Saha, and Satyesh C. Roy

### Abstract

This protocol describes an efficient and rapid method for large-scale multiplication of *Cordyline terminalis* in a cost-effective manner. Actively growing shoot tips were selected as explants. Murashige and Skoog (MS) basal medium was supplemented with different plant growth regulators at various developmental stages of *C. terminalis*. The highest percentage of regeneration ( $95 \pm 2.8$ ) and average number of shoot buds ( $60.2 \pm 4.4$ ) per explant were obtained in medium containing 80 mg/L adenine sulfate ( $\text{AdSO}_4$ ), 2 mg/L 6-benzyladenine (BA), and 0.1 mg/L indole-3-acetic acid (IAA). Thousands of micropropagated plants were produced within 4–5 months using this protocol.

**Key words:** Adenine sulfate, *Cordyline terminalis*, Micropropagation, Ornamental plants, Shoot apex explants

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### 1. Introduction

*Cordyline terminalis*, a native of Southeast Asia belonging to the family Agavaceae, is considered to be one of the most economically important ornamental indoor plants for its beautiful multicolored foliage. The economic value of ornamental plants has raised perceptibly worldwide and is still increasing to meet the steady demand of floriculture industry. To maintain the genetic stability of the variegated leaves, conventionally this cultivar is propagated by rhizomes or by terminal stem (tips) cuttings and is planted directly in pots. This method of vegetative propagation has reduced the interest due to very slow growth of the plant (1) and eventually long delay in commercial distribution. Moreover, there are newly introduced cultivars which do not develop rhizome (2).

Micropropagation is the most extensively used and a routinely applied technique to mass propagate plants where the rate of conventional multiplication is otherwise very slow (3–6). Additionally,

micropropagation gives the opportunity to produce a large number of clean and healthy uniform plants in small space in a timely manner and also reduces the need of mother plant (7).

Here we describe a standardized, rapid, and efficient method of micropropagation for large-scale production of *C. terminalis* plants.

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## 2. Materials

### 2.1. Surface Sterilization of Source Material

1. Tap water.
2. Absolute ethanol and 70% (v:v) ethanol.
3. 0.01% (v:v) Tween 20 (Sigma, St. Louis, USA).
4. 0.1% (w:v) Mercuric chloride ( $\text{HgCl}_2$ ) (Qualigen, India) (see Note 1).
5. Autoclaved double distilled water in 500 mL screw-capped Duran glass bottles, (Schott AG, Mainz, Germany).
6. Measuring cylinders, 100 mL capacity (Borosil, India).
7. Beakers, 250 mL capacity (Borosil).
8. Autoclaved jam bottles (Sadana Brothers, India).
9. Autoclaved Whatman No.1 filter paper in Petri dishes (50 mm diameter; Borosil).
10. Tissue culture facilities—Instruments (scalpel, forceps, spirit lamp to flame sterilize instruments), laminar air flow, culture room.
11. Two to three year-old ex vitro grown *C. terminalis* plants as source of explants.

### 2.2. Culture Media

1. Murashige and Skoog (MS) (8) medium containing different concentrations of various plant growth regulators is used for this protocol. Formulation of MS medium stock solutions (see Note 2) and plant growth regulators are given in Tables 1 and 2, respectively.
2. Measuring cylinders, 500 mL and 1 L capacity (Borosil).
3. Beakers, 500 mL and 1 L capacity (Borosil).
4. Erlenmeyer flasks, 250 mL capacity (Borosil).
5. Culture tubes, 12 × 100 mm (Borosil).
6. General tissue culture laboratory facilities—Equipments (analytical scale, autoclave, distilling unit, magnetic stirrer with hot plate, microwave oven, pH-meter).

### 2.3. Acclimatization of Regenerated Plants to Ex Vitro Conditions

1. Plastic net pots (5 cm) and plastic pots (15 cm).
2. Soilrite (Keltech Energies Ltd. India).

**Table 1**  
**Stock solution compositions of Murashige and Skoog (MS)**  
**medium (8)**

Stock solution 1 (major salts)	Concentration (20×) (g/L)
$\text{NH}_4\text{NO}_3$	33
$\text{KNO}_3$	38
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	8.8
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	7.4
$\text{KH}_2\text{PO}_4$	3.4
Stock solution 2 (minor salts)	Concentration (200×) (g/L)
KI	0.166
$\text{H}_3\text{BO}_3$	1.24
$\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$	4.46
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	1.72
$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	0.05
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.005
$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	0.005
Stock solution 3 (Fe-EDTA)	Concentration (200×) (see Note 2) (g/L)
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	5.57
$\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$	7.45
Stock solution 4 (myo-inositol)	Concentration (1,000×) (mg/mL)
Myo-inositol	50
Stock solution 5 (vitamins)	Concentration (1,000×) (mg/mL)
Thiamine-HCl	0.1
Nicotinic acid	0.5
Pyridoxine-HCl	0.5

**Table 2**  
**Stock solutions of plant growth regulators**

Plant growth regulators	Concentration (mg/mL)
Adenine sulfate ( $\text{AdSO}_4$ )	2
6-benzyladenine (BA)	0.5
Indole-3-acetic acid (IAA)	0.5
Indole-3-butyric acid (IBA)	0.5

See Note 3 for plant growth regulators stock solutions preparation

### 3. Methods

#### 3.1. Preparation and Sterilization of Culture Media

1. Prepare full strength MS basal medium according to Table 3 by adding stock solutions of Table 1.
2. Make up the final volume of media with double distilled water after dissolving the sucrose (Hi-media, India). Add stock solutions and sucrose according to the strength of media.
3. Supplement media with different concentrations of various plant growth regulators such as adenine sulfate ( $\text{AdSO}_4$ ), 6-benzyladenine (BA), indole-3-acetic acid (IAA), and indole-3-butyric acid (IBA) (Sigma) (Table 4) by using the stock solutions (Table 2) according to the developmental stages (see Note 3).
4. Adjust the pH of all the media to 5.8 using 0.1 N NaOH or 0.1 N HCl (see Note 4).
5. Add required amount of agar (Hi-media, India) to make the solid media (Table 3) after adjusting the pH.
6. Melt the agar by heating media. Dispense approximately 20 mL shoot initiation and multiplication (SIM) medium in culture tubes and 100 mL media in 250 mL Erlenmeyer flasks or jam bottles for shoot elongation and root initiation, respectively.
7. Provide a support of filter paper bridge in the culture tubes for liquid root elongation (RE) medium (Table 4).
8. Autoclave media and all other required glass apparatus and instruments at  $121^\circ\text{C}$  and 15 lbs/in<sup>2</sup> for 15 min after proper plugging and wrapping.
9. Incline the SIM medium immediately after autoclaving to prepare slants.

**Table 3**  
**Preparation of MS basal medium**

Components	Amount required for 1 L of medium
Stock solution 1 (major salts)	50 mL
Stock solution 2 (minor salts)	5 mL
Stock solution 3 (Fe-EDTA)	5 mL
Stock solution 4 (myo-inositol)	2 mL
Stock solution 5 (Vitamins)	1 mL
Sucrose	30 g
Agar	6 g

**Table 4**  
**Observation for optimum production of plants on media fortified with different growth regulators**

Observation	Results <sup>a</sup>	Duration of culture (days)	Name of media	Type of media	Concentration of growth regulator (mg/L)			
					AdSO <sub>4</sub>	BA	IAA	IBA
Percentage of explants regeneration	95 ± 2.8	15	SIM	MS (solid)	80	2	0.1	0
Average number of shoot buds	60.2 ± 4.4	25–30	SIM	MS (solid)	80	2	0.1	0
Average length of elongated shoots (cm)	3.8 ± 0.1	7–14	SE	½ MS (solid)	0	1	0	0
Average number of roots per plantlet	4.8 ± 0.5	14–20	RI	½ MS (solid)	0	0	0	0.5
Average length of root (cm)	4.5 ± 0.2	10–14	RE	½ MS (liquid)	0	0	0	0

SE shoot elongation medium; SIM shoot initiation and multiplication medium; RI root initiation medium; RE root elongation medium

<sup>a</sup>Data presented as the mean value ± standard error

### 3.2. Surface Sterilization of Explants

1. Cut about 1 cm long apical part of shoot apex of *C. terminalis* plants. Remove sheathing leaves around the apical meristem, and wash shoot apices thoroughly under tap water (see Note 5).
2. For surface sterilization, first rinse the shoot apices, with 0.01% (v:v) tween-20 solution for 10 min. Then wash 2–3 times with sterile distilled water to remove soapy traces of tween-20.
3. Under laminar air flow, treat explants with 0.1% (w:v) HgCl<sub>2</sub> for 10 min in an autoclaved jam bottle, followed by 3–4 washes in sterile distilled water to remove traces of HgCl<sub>2</sub>.
4. Remove excessive water from the explants with sterile filter paper by placing in Petri dishes. By using a sterile scalpel, remove the exposed tissue of surface sterilized explants leaving the central cylinder of apical meristem. Before culture on the medium, cut explants into 2–3 mm small pieces.

### 3.3. Culture and Maintenance of Explants

1. During every culture and subculture, follow the routine aseptic steps of a tissue culture laboratory like swabbing hands and wiping the inside of laminar air flow with 70% (v:v) ethanol; switch on the UV lamp 30 min before starting culture.

2. Place a piece of explants with a sterile forceps on the surface of the slant of SIM medium (Table 4) in each culture tube (see Note 6).
3. Maintain all the cultures under  $39 \mu\text{mol}/\text{m}^2/\text{s}$  white fluorescent light (Philips, India) for 16 h photoperiod at  $25 \pm 2^\circ\text{C}$  with 55–60% relative humidity until explants gradually swell, become enlarged in size, and turn light greenish.
4. Make shoot elongation (SE) medium (Table 4) after 20 days of initial culture when primary microshoots begin to appear (Fig. 1a).

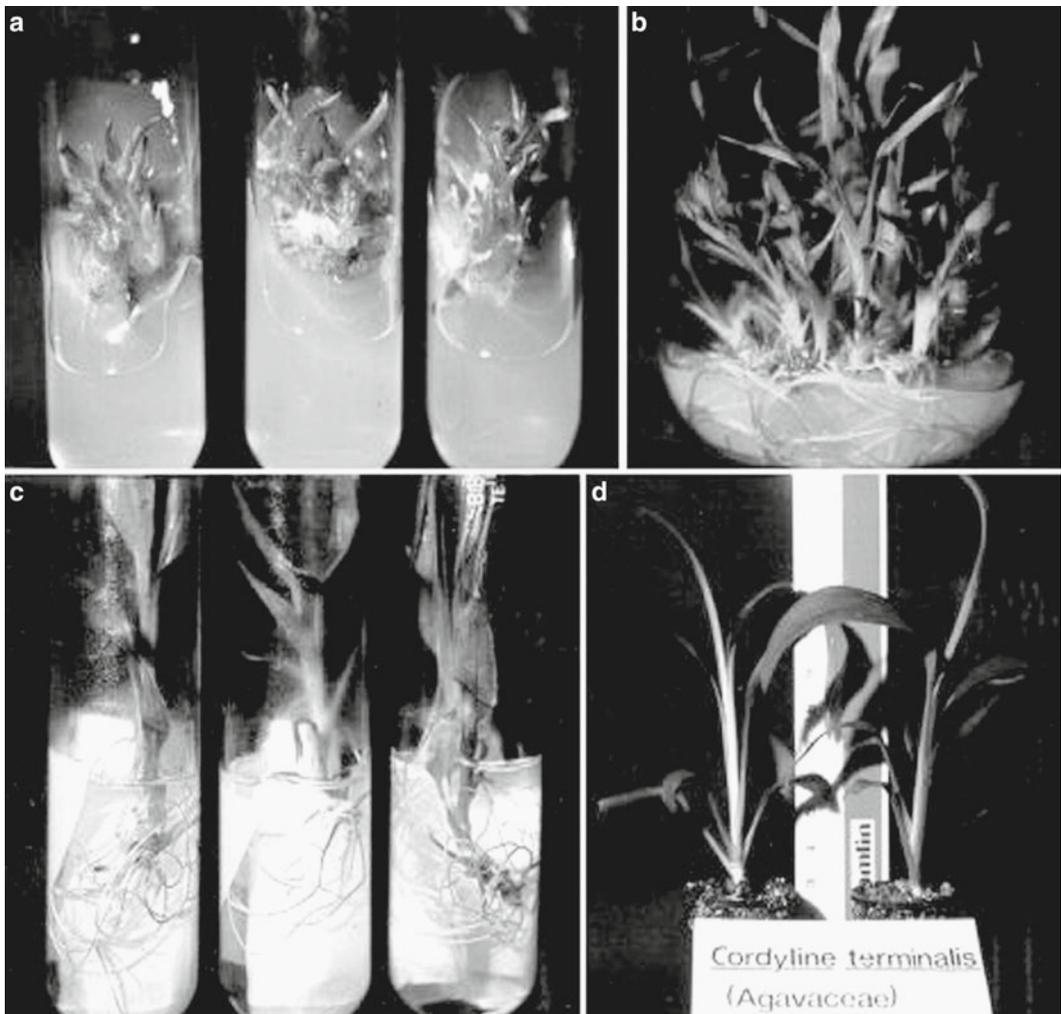


Fig. 1. Different stages of micropropagation of *C. terminalis* from shoot apex meristem. (a) Multiple shoot bud formation from shoot apex explant after 20 days in culture. (b) Numerous shoots with profuse leaves and roots on root initiation (RI) medium. (c) Regenerated plants in half strength MS basal liquid (RE) medium. (d) Micropropagated plants, 20 days after transplanting in soil.

5. Excise and subculture primary microshoots (0.75–1.0 cm long) on SE medium containing half strength MS basal medium supplemented with 1 mg/L BA in 250 mL Erlenmeyer flasks or in jam bottles. Eliminate callus tissues, if any, carefully from the base of newly formed shoots before subculture and place shoot clusters well separated on SE medium. Subculture the swelled explants back in a fresh slant of SIM medium for subsequent regeneration (see Note 7).
6. Separate the elongated shoots from the clusters after 10–14 days and subculture individually on fresh SE medium with same composition in 250 mL Erlenmeyer flasks or in jam bottles (see Note 8).

### **3.4. Root Development on Regenerated Shoots In Vitro**

1. Transfer shoots (3–5 cm long) with two or more leaves to root initiation (RI) medium amended with half strength MS basal medium fortified with 0.5 mg/L IBA (Table 4) in 250 mL Erlenmeyer flasks or sometimes in jam bottles for root induction (Fig. 1b) (see Note 9).
2. Transfer plantlets with well-developed roots from solid RI medium to half strength MS basal liquid RE medium with a support of filter paper bridge for 14 days (Fig. 1c) (see Note 10).
3. Transfer plantlets with profuse roots successively to one-fourth and one-eighth strength MS basal liquid RE media for 14 days in each, respectively (see Note 11).
4. Finally transfer the rooted plants in one-eighth strength of MS major and minor salt solutions (Table 1) for 7 days.

### **3.5. Acclimatization of In Vitro Plants to Ex Vitro Conditions**

1. Transfer all the rooted plants to net pots containing autoclaved soilrite. At this stage, water plants with one-eighth strength of MS major and minor salt solutions.
2. Maintain the regenerated plants in growth room for 7–10 days inside jam bottles containing water so that the lower 1 cm of the net pots remain immersed (see Note 12). Leave the lid of jam bottles open for 1 h to whole day gradually increasing the time from the third day of transfer to net pots.
3. Finally transplant plants in 15 cm diameter pots in soil and maintain in the glasshouse for 14 days and then to the field (Fig. 1d).

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## **4. Notes**

1. Dissolve 100 mg  $\text{HgCl}_2$  in 1 mL absolute ethanol and make up the volume with water to prepare 100 mL 0.1%  $\text{HgCl}_2$  solution.
2. Dissolve 745 mg  $\text{Na}_2\text{EDTA}\cdot 2\text{H}_2\text{O}$  in 75 mL boiling double distilled water and add 557 mg  $\text{FeSO}_4\cdot 7\text{H}_2\text{O}$ . Keep stirring on

a hot plate magnetic stirrer for at least 1 h at 100°C until the color of the solution turns to golden yellow. Finally make up the volume to 100 mL and store in amber-colored bottle at 4°C in the refrigerator. Except plant growth regulators, all other stock solutions are prepared by dissolving required amount of reagents in double distilled water and finally stored at 4°C.

3. Stock solutions of plant growth regulators should be prepared in small amount and stored at 0°C. Growth regulators should be stored maximum for 7 days. Add few drops of concentrated HCl to dissolve 20 mg AdSO<sub>4</sub> in 10 mL double distilled water before making up the final volume. Always prepare fresh AdSO<sub>4</sub> solution just before media preparation. Similarly, dissolve 5 mg BA in 1 mL 1 N HCl and make up volume to 10 mL with double distilled water. Dissolve 5 mg IAA or IBA in 1 mL absolute ethanol and make up volume to 10 mL.
4. Adjust the pH with 0.1 N NaOH or 0.1 N HCl with continuous stirring. Addition of AdSO<sub>4</sub> increases the pH too high. Considering that keep the volume of media enough low, at least 10–15 mL so that adequate HCl can be added to adjust the pH at 5.8.
5. We selected shoot apex meristem as an explant, since the organized meristems do not undergo much genetic change during cell division (9).
6. We tested various concentration of AdSO<sub>4</sub> (20–100 mg/L) and BA (0.5–5 mg/L) in combination with auxin IAA (0.05–1 mg/L) for shoot bud induction (6). Application of AdSO<sub>4</sub> (80 mg/L), BA (2 mg/L) with low concentration of IAA (0.1 mg/L) showed highest regeneration rate (95 ± 2.8%) and the highest number of shoot buds (60.2 ± 4.4) per explant (Table 4). Approximately after 7 days of culture, explants became enlarged in size, swelled, turned light greenish, and gradually within 20 days shoot buds began to appear. The high concentrations of BA and IAA were quite inhibitory on shoot bud formation. With the higher concentration of (0.5 mg/L) IAA, shoot apex explants swelled and turned into a yellowish hyaline mass of calli within 20–25 days, which later turned brown (6).
7. Repeated subculture of shoot clusters in the same medium accelerated the formation of shoots in large numbers. The shoot multiplication could be repeated up to 12–15 multiplication cycles with normal morphogenesis without returning to the original explant source.
8. Sometimes microshoots started rooting in the SE medium after being separated from clusters, but those roots were not well developed, so we cut those roots before transferring the individual microshoot in RI medium.

9. We tried different concentrations (0.5–2 mg/L) of IAA or IBA or both in RI media. Of these, IBA (0.5 mg/L) was the most effective in inducing roots and showed normal rooting, whereas IAA produced deformed roots (6).
10. All rooted microshoots showed profuse growth and elongation in liquid half strength of MS basal medium.
11. It is very important to gradually eliminate sucrose from the liquid medium so that plants photosynthesize independently.
12. Immersing the lower part of the pots in water helped roots to come out of the net pot quickly and grow well into soil.

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## Micropropagation of African Violet (*Saintpaulia ionantha* Wendl.)

Mukund Shukla, J. Alan Sullivan, Shri Mohan Jain,  
Susan J. Murch, and Praveen K. Saxena

### Abstract

Micropropagation is an important tool for rapid multiplication and the creation of genetic variability in African violets (*Saintpaulia ionantha* Wendl.). Successful in vitro propagation depends on the specific requirements and precise manipulation of various factors such as the type of explants used, physiological state of the mother plant, plant growth regulators in the culture medium, and growth conditions. Development of cost-effective protocols with a high rate of multiplication is a crucial requirement for commercial application of micropropagation. The current chapter describes an optimized protocol for micropropagation of African violets using leaf explants obtained from in vitro grown plants. In this process, plant regeneration occurs via both somatic embryogenesis and shoot organogenesis simultaneously in the explants induced with the growth regulator thidiazuron (TDZ; *N*-phenyl-*N'*-1,2,3-thiazol-5-ylurea). The protocol is simple, rapid, and efficient for large-scale propagation of African violet and the dual routes of regeneration allow for multiple applications of the technology from simple clonal propagation to induction or selection of variants to the production of synthetic seeds.

**Key words:** African violet, *Saintpaulia ionantha*, Micropropagation, Somatic embryogenesis, Organogenesis, Regeneration, Root initiation, Thidiazuron

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### 1. Introduction

African violet (*Saintpaulia ionantha* Wendl.; Gesneriaceae) is a commercially important indoor ornamental plant species highly valued in many parts of the world. Thousands of *Saintpaulia* cultivars have been selected for plant size, floral colors, leaf shapes and pattern, growth, uniform flowering, and better performance as a house plant. While these plants are propagated most commonly by vegetative leaf cuttings, in vitro methods are widely used both for large-scale production and the introduction of genetic variability for new cultivar development. Micropropagation of African violet

from various types of explants, including leaf discs, petioles, petals, and anthers has been reported by various researchers (1–8). Regeneration of African violet has been achieved through direct differentiation of shoots from different explants (9–12) as well as an indirect mode of organogenesis with an intermediate callus phase (13, 14). African violet leaf and petiole tissues have been shown to regenerate via organogenesis and somatic embryogenesis (7) following induction with thidiazuron (TDZ). TDZ is a highly potent plant growth regulator (PGR) known to stimulate a number of different physiological responses in plants including *de novo* plant regeneration (15–22). The specific mode of action of TDZ in plant tissues remains undetermined and a dual role for the PGR as synthetic stimulator of both auxin and cytokinin metabolism continues to be proposed (8, 17, 23, 24).

In this chapter, we describe an efficient protocol for micro-propagation of African violet based on the use of TDZ as an inductive signal of regeneration and axenic shoot cultures (ASC) as the source of the explants. Various stages of the development of the plantlets in this process include: (1) initiation of ASC, (2) micro-propagation via simultaneous shoot organogenesis and somatic embryogenesis from the explants of ASC, (3) rooting and root growth of the regenerated shoots and somatic embryo-derived plantlets, and (4) acclimatization and greenhouse transplant of regenerated plantlets.

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## 2. Materials

### **2.1. Surface Sterilization of Source Material**

1. Tap water.
2. Ethanol 70% (v:v).
3. Autoclaved distilled water; 250 mL aliquots in 500 mL screw capped bottles.
4. Commercial bleach solution (e.g., “CLOROX®” bleach; 5.5% (v/v) NaClO), diluted 2:10 (v:v) with autoclaved distilled water.
5. Tween 20 (Fisher BioReagents, USA).
6. Magnetic stirrer, magnetic bar, 600 mL beaker (autoclaved).
7. Instruments (scalpel, forceps, glass bead sterilizer), laminar flow bench.
8. Media preparation and tissue culture facilities, culture room.
9. Potted plants of African violet cv. Benjamin obtained from a commercial greenhouse (Harster Greenhouses Inc., Dundas, ON, Canada).

**Table 1**  
**Murashige and Skoog (MS) basal medium with vitamins**

Constituents		Chemical formula	Concentration (mg/L)
Macroelements	Ammonium nitrate	$\text{NH}_4\text{NO}_3$	1,650
	Potassium nitrate	$\text{KNO}_3$	1,900
	Calcium chloride	$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	332.2
	Magnesium sulfate	$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	180.7
	Potassium phosphate	$\text{KH}_2\text{PO}_4$	170
Microelements	Potassium iodide	KI	0.83
	Boric acid	$\text{H}_3\text{BO}_3$	6.2
	Manganese sulfate	$\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$	16.9
	Zinc sulfate	$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	8.6
	Molybdic acid (sodium salt)	$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	0.25
	Cupric sulfate	$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.025
	Cobalt chloride	$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	0.025
Iron	Ferrous sulfate	$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	27.80
	$\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$		37.26
Vitamins	Myo inositol		100
	Glycine		2.0
	Nicotinic acid		0.5
	Pyridoxine-HCl		0.5
	Thiamine-HCl		0.1

## 2.2. Culture Media

1. Culture media contained salts and vitamins according to Murashige and Skoog (25) and PGRs viz. BA, NAA, and TDZ (Sigma-Aldrich Co., St Louis, MO, Difco, Detroit, MI and Phytotechnology, KS, USA).
2. The media formulation are listed in Tables 1 and 2 for
  - (a) Establishment of in vitro culture from leaf and petiole explants.
  - (b) Regeneration from ASC.
  - (c) Root induction on regenerated shoots.
3. Petri dishes (100 × 15 mm; Phytotechnology, KS, USA).
4. Magenta boxes (3 × 3 × 4"; Phytotechnology, KS, USA).
5. Other glassware (Fisher Scientific, USA).

## 2.3. Acclimatization of Regenerated Plants in Greenhouse

1. Tap water.
2. Plastic pots (6 in.; ITML Horticultural Products, Middlefield, OH, USA).

**Table 2**  
**Preparation and storage of different plant growth regulators (PGR) used for in vitro propagation of African violet**

Growth regulator	Molecular weight	Preparation and storage		
		Solvent	Diluents	Storage (°C)
BAP	225.3	1 N NaOH	Water	0–5
NAA	186.2	1 N NaOH	Water	0–5
TDZ	220.2	DMSO	Water	0–5

3. Plastic trays with cover (72 cells; ITML Horticultural Products, Middlefield, OH, USA).
4. Soil-less mix prepared by combining ProMix™ and Perlite (Therm-O-Rock East, Inc., New Eagle, PA, USA) (1:1 by volume).
5. Sunshine® professional growing mix (Sun Gro Horticulture, Vancouver, BC, Canada).
6. Plant growth chamber.
7. Greenhouse.

### 3. Methods

#### 3.1. Preparation of Culture Media

The composition of basal medium containing the mineral components of MS medium supplemented with vitamins (25) is provided in Table 1. PGRs and the conditions of their storage are listed in Table 2. The medium is solidified with 2.5 g/L Gelrite (Sigma, USA) and the pH of the media adjusted to 5.7 before autoclaving at 121°C and 1.1 kg/cm<sup>2</sup> for 20 min. The volumes of the culture medium dispensed in each Petri dish and Magenta box are 15 mL and 50 mL, respectively.

#### 3.2. Establishment and Maintenance of Source Material

1. Maintain the plants of *S. ionantha* Wendl. cv. Benjamin in 6 in. pots filled with an artificial soil mix (Sunshine® professional growing mix) in the greenhouse. The temperature in the greenhouse should be within a range of 20–24°C with a 16/8 h photoperiod (day/night). The light intensity should be at 80–85 μmol/s/m<sup>2</sup> and the relative humidity at 55–60%. Select the healthy plants with fully expanded leaves for initiating micropropagation. Use the leaf disc or petiole explants from the green house-grown plants to generate ASC.
2. Prepare the disinfectant bleach solution by diluting the commercial bleach (20 mL of 5.4% sodium hypochlorite), with

80 mL sterile distilled water, and adding three drops of the wetting agent “Tween 20.” Disinfect leaf tissue by placing 2–4 leaves in a 250 mL sterile beaker and rinse in 70% ethanol for 1 min. Decant ethanol and gently pour the diluted bleach solution on the leaves to submerge completely for surface sterilization. Stir the contents gently for 15–20 min. Rinse the leaves five times with sterile de-ionized distilled water to remove the bleach. Each rinse should be for a period of 3–4 min.

3. For preparing the leaf explants, cut the mature leaves (4–6 cm long) into rectangular discs, approximately 1.5 × 1 cm in size, using a scalpel. Remove the petiole explants and section them transversely (<0.25 mm thick) with a sharp scalpel. Keep the explants in sterile water prior to culture.
4. Culture the leaf disc and petiole explants on MS medium containing MS salts, vitamins, 3% sucrose and a growth regulator combination of 1 μM benzyladenin (BA), and 1 μM naphthalene acetic acid (NAA) or 5 μM TDZ (*N*-phenyl-*N'*-1,2,3-thiadiazol-5-ylurea). Culture the leaf disc explants in test tubes (5 × 15 cm) or magenta boxes whereas the petiole explants in Petri dishes. Place the leaf explant with the abaxial surface in contact with the medium and ensure that the orientation of the petiole explants is such that the surface farthest from the leaf blade is exposed to the culture medium. Maintain the cultures in a growth room at 24°C under a 16 h photo period (50 μmol/s/m<sup>2</sup>) provided by cool white fluorescent lamps.
5. De novo shoots and somatic embryos will develop within 3–4 weeks (Fig. 1a–d) and well-formed shoots after 6 weeks (Fig. 1e). Somatic embryos develop from the epidermal cells surrounding the petiole slices and are loosely attached to the maternal tissues by a suspensor of transparent cells and are easily removed with forceps (Fig. 1b, c). The explants cultured with cut surface closest to the leaf blade exposed to the medium may not form somatic embryos or produce a few embryos infrequently and with an arrested development.
6. Remove the shoots (Fig. 1d) with the help of scalpel and forceps and subculture in Magenta box containing MS basal medium (50 mL) with 3% sucrose. These ASC are maintained by subculturing on basal medium at 4-weeks interval (Fig. 1f).

### 3.3. Micropropagation

1. Regeneration of African violet via shoot organogenesis or somatic embryogenesis has been reported from various explants. The protocol described below uses the ASC as the primary source of explants for micropropagation. Remove fully expanded leaves from ASC and place in sterile Petri dish containing a few drops of sterile water to prevent desiccation. Cut the leaves into approximately 1 cm segments using a sharp scalpel. Culture excised leaves on the induction medium containing the



Fig. 1. Micropropagation of African violet. (a) Regeneration from leaf explants of greenhouse grown plants. (b) Development of somatic embryos from epidermal region of petiole explants. (c) A somatic embryo loosely attached to the explant. (d) Shoot and somatic embryos at various stages of growth and elongation. (e) Regenerating shoots from leaf disc explants. (f) An axenic shoot culture (ASC) with well-formed leaves. (g) A mature in vitro grown plant with well-formed flowers. (h) Greenhouse transplant of regenerated plantlets. (i) A variegated chimera of African violet originating from regenerated plantlets.

ingredients of MS medium (Table 1) and various concentrations of TDZ (Table 3).

2. Culture five explants in each Petri dish containing 15 mL medium. Ensure that the abaxial surface of the leaf is in contact with the culture medium. Seal the Petri dishes with Parafilm and place the cultures under the same growth conditions as described earlier for ASC.
3. Frequency of regeneration is affected by the type and duration of exposure of explants to the induction medium. Both the concentration of TDZ and the duration of exposure must be optimized for each cultivar or genotype. Select the optimum

**Table 3**  
**Effect of the concentration of TDZ and the duration of exposure (3 or 9 days) on average number of regenerants per explant**

TDZ concentration ( $\mu\text{M}$ )	Number of regenerants per explant	
	3 Days	9 Days
0.0	0	0
1.0	6–10	8–12
2.0	19–26	20–23
5.0	16–21	15–18
10.0	15–21	10–18

concentration of TDZ for inducing de novo development of shoots and somatic embryos (Table 3). For the cv. Benjamin, the optimum concentration of TDZ is 1–5  $\mu\text{M}$  with an exposure period of 9 days to produce a significantly higher number of regenerants (shoots and embryo) compared to other concentrations (Table 3). Concentrations of TDZ higher than 5  $\mu\text{M}$  increases the number of somatic embryos compared to shoots although the total number of regeneration remains similar.

- Transfer the explants induced on TDZ for 9 days onto the MS basal medium without growth regulators in Magenta boxes each containing 50 mL medium for 2–3 regenerating explants.
- Multiple shoots and somatic embryos develop after 3–5 weeks on the explants induced with 5  $\mu\text{M}$  TDZ in a similar manner observed for the greenhouse-grown petioles (Fig. 1a–e). The somatic embryos are loosely attached to the source tissue and the shoot can also be easily excised for further development into plantlets. Carefully remove regenerating shoots and germinated embryos and transfer on to MS basal medium for further expansion and growth into well-formed shoots in about 4–5 weeks. The stages of shoot regeneration and somatic embryo development resemble those shown in Fig. 1a–e. Various steps of African violet micropropagation are shown in Fig. 2.

### **3.4. Rooting and Plantlet Development**

Separate the shoots and germinated somatic embryos with or without visible roots. Transfer these shoots (approximately 4.0 cm long) to MS medium supplemented with 0.60  $\mu\text{M}$  NAA. A well-formed root system will develop after 1–2 weeks and these plantlets are easily grown to mature flowering plants (Fig. 1g) following transplant in the greenhouse.

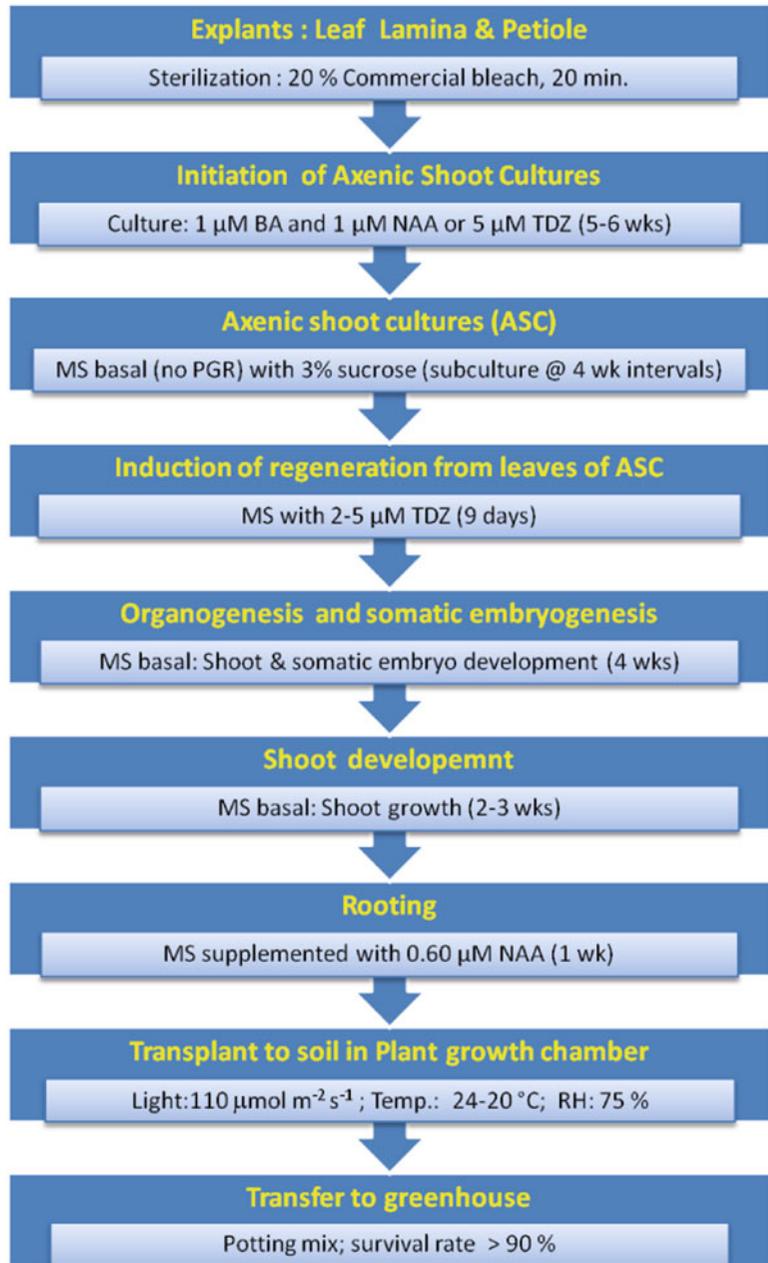


Fig. 2. Protocol for micropropagation of African violet (*Saintpaulia ionantha* Wendl.).

### 3.5. Acclimatization and Growth in the Greenhouse

- 1 Acclimatize the regenerated, rooted plantlets before transfer to the greenhouse. Gently remove the plantlets from the culture medium and carefully wash with running tap water ensuring minimum damage to the tissue. Transplant the clean plants into 72 cell trays containing a soil-less mix prepared by combining ProMix™ and perlite in equal volumes. Cover the trays

with transparent plastic covers and place in the growth chambers set at 16 h light (24°C) and 8 h dark (20°C) cycle and 95% relative humidity. Reduce the relative humidity in the chambers every week by 5% for 3 weeks and thereafter maintain consistently around 80%. Remove the plastic covers at the end of the third week.

- 2 Transfer the flats to the greenhouse at the end of the fourth week and transplant into 6-in. standard pots filled with Sunshine® professional growing mix (Fig. 1h). Grow the potted plants under the typical greenhouse conditions to evaluate their growth performance and flowerings. Occasionally, spontaneous variants such as chimeras with variegated leaves (Fig. 1i) are seen in regenerated progeny, although at a low frequency.

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#### 4. Notes

1. For micropropagation of African violet, the most commonly used explants are leaf discs and petioles. The ASC are a better source of explants due to their consistent availability, physiological uniformity, and preconditioning in vitro. In addition, the use of ASC eliminates the need of surface sterilization. However, the process of establishing ASC can also be effectively used for direct micropropagation from the greenhouse-grown plants, and presumably from the plants grown in natural environments. Both the leaf discs as well as the petioles can be used as explants, but the frequency of regeneration would vary from one cultivar to another. Regeneration efficiency of leaf and petiole explants from in vitro and the greenhouse-grown plants also varies (7, 8) and requires optimization of PGR concentrations.
2. This micropropagation protocol of African violet involves the induction of shoot organogenesis and somatic embryogenesis on the same explant. Somatic embryos develop frequently at higher concentrations (5–10 μM) compared to shoots at low concentrations (1–2 μM) of TDZ. Somatic embryogenesis is unique characteristic of plant cells for obtaining a large number of genetically similar plants in a short time. The somatic embryo production in African violet is of commercial importance as this is a high-value crop with potential problems of somaclonal variation and chimeric plants. Additionally, regeneration via somatic embryogenesis is advantageous for genetic manipulation and propagation of this species due to the single cell origin and bipolar growth habit of the embryos. Somatic embryogenesis also facilitates encapsulation of embryos with synthetic gels to develop artificial seeds and cryopreservation

for long-term germplasm conservation. Regardless of the nature of regeneration, the micropropagation protocol described here can be further adapted for large-scale propagation using bioreactors.

3. TDZ is a unique and highly potent growth regulator which can be substituted for both the auxin and cytokinin requirements of organogenesis and somatic embryogenesis in several species (7, 8, 17, 26). The TDZ-induced somatic embryogenesis of African violet will also provide a system for the investigation of the biochemical and molecular factors controlling the development of somatic embryos including the transport of interacting endogenous and exogenous compounds.

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## Acknowledgement

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## Micropropagation of *Iris* sp.

Sladana Jevremović, Zoran Jeknić, and Angelina Subotić

### Abstract

Irises are perennial plants widely used as ornamental garden plants or cut flowers. Some species accumulate secondary metabolites, making them highly valuable to the pharmaceutical and perfume industries. Micropropagation of irises has successfully been accomplished by culturing zygotic embryos, different flower parts, and leaf base tissues as starting explants. Plantlets are regenerated via somatic embryogenesis, organogenesis, or both processes at the same time depending on media composition and plant species. A large number of uniform plants are produced by somatic embryogenesis, however, some species have decreased morphogenetic potential overtime. Shoot cultures obtained by organogenesis can be multiplied for many years. Somatic embryogenic tissue can be reestablished from leaf bases of in vitro-grown shoots. The highest number of plants can be obtained by cell suspension cultures. This chapter describes effective in vitro plant regeneration protocols for *Iris* species from different types of explants by somatic embryogenesis and/or organogenesis suitable for the mass propagation of ornamental and pharmaceutical irises.

**Key words:** Cell suspension, Irises, Leaf base, Organogenesis, Somatic embryogenesis, Zygotic embryos

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### 1. Introduction

Irises (*Iris* sp.) are perennial monocotyledonous plants, widely distributed throughout the temperate regions of the Northern Hemisphere. Many species of the genus *Iris* are cultivated for their beautiful and attractive flowers. Some *Iris* plants produce secondary metabolites that are used in the pharmaceutical, cosmetic, and perfume industries (1, 2). Traditionally, irises are propagated vegetatively either by bulbs or splitting of rhizomes because of their allogamous nature. These propagation methods are inefficient and slow, especially for large-scale production of plants of new, commercially valuable, cultivars. Micropropagation of irises began during sixth decade of the last century when the first virus-free plants were obtained through meristem culture (3). Over time, several protocols for in vitro induction of morphogenesis in many cultivated, endemic, or endangered *Iris* species were reported (4–7). In monocots,

including *Iris*, one of the most important factors for the successful induction of in vitro morphogenetic response is the source of explants. Apparently, cell differentiation in monocots starts early and proceeds rapidly limiting the explant source to only meristematic region and adjacent tissues. To date, the successful micropropagation of irises has been reported for 18 different species (8). Plant regeneration was achieved from zygotic embryos (immature and mature), leaf bases, flower parts, bulbs, and root cultures (9–12).

This chapter describes efficient protocols for the micropropagation of irises from zygotic embryos and leaf base cultures. These protocols could be applied for large-scale production of several *Iris* sp., which are valuable to ornamental and pharmaceutical industries.

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## 2. Materials

### 2.1. Plant Material

1. Seeds or potted *Iris* plants (see Note 1).

### 2.2. Instruments

1. Laboratory and tissue culture facilities (autoclave, electronic balances, centrifuge, pH meter, water purifier, pipets, laminar flow hood).
2. Laboratory vessels (Petri dishes, bottles, Magenta boxes or glass jars, test tubes, cotton/foam plugs).
3. Tissue culture instruments (scalpel, forceps, spirit or mixed gas burner).
4. Culture room or a controlled environment growth chamber [ $24 \pm 2^\circ\text{C}$  temperature condition and 16-h light/8-h dark photoperiod with  $50 \mu\text{mol}/\text{m}^2/\text{s}$  PPFD (Photosynthetic Photon Flux Density) or complete darkness].

### 2.3. Surface Sterilization of Plant Material

1. Tap water.
2. Commercial bleach solution (4% (v/v) NaClO) diluted to 20% with sterile water.
3. Sterile distilled or deionized water (autoclaved or filter-sterilized); 750 mL aliquots in 1 L screw capped bottles.
4. Liquid Antimicrobial Hand Soap (The Dial Corporation, Scottsdale, AZ, USA).

### 2.4. Chemicals for Culture Media

1. All inorganic chemicals for preparation of Murashige and Skoog mineral solution (MS) (13). The chemicals required for preparing stock solutions are listed in Table 1.
2. Organics (nicotinic acid, panthotenic acid, adenine sulfate, casein hydrolysate), amino acids (proline, tyrosine), vitamins: Thiamine-HCl (vitamin B<sub>1</sub>), Pyridoxine-HCl (vitamin B<sub>6</sub>), and plant growth regulators: 6-Benzyladenine (BA); Kinetin (KIN);  $\alpha$ -Naphthalenacetic acid (NAA); 2,4-Dichlorophenoxyacetic acid (2,4-D); and Thidiazuron (TDZ) (Table 2).

**Table 1**  
**Composition of Murashige and Skoog mineral solution (13)**

Code	Components (mg) <sup>a</sup>		Final volume of stock solution (mL)	Volume of stock solution per 1 L of final medium (mL)
I	NH <sub>4</sub> NO <sub>3</sub>	6,600	800	20
	KNO <sub>3</sub>	7,600		
II	MgSO <sub>4</sub> ·7H <sub>2</sub> O	1,480	400	10
	MnSO <sub>4</sub>	892		
	ZnSO <sub>4</sub> ·7H <sub>2</sub> O	344		
	CuSO <sub>4</sub> ·5H <sub>2</sub> O	1		
III	CaCl <sub>2</sub> ·2H <sub>2</sub> O	1,760	400	10
	KI	332		
	CoCl <sub>2</sub> ·6H <sub>2</sub> O	1		
IV	KH <sub>2</sub> PO <sub>4</sub>	6,800	400	10
	H <sub>3</sub> BO <sub>3</sub>	248		
	NaMoO <sub>4</sub> ·2H <sub>2</sub> O	10		
V	FeSO <sub>4</sub> ·7H <sub>2</sub> O	2,228	400	5
	Na <sub>2</sub> EDTA	2,976		

<sup>a</sup>Dissolve separately before mixing in the final stock

**Table 2**  
**Composition of organics and growth regulators used for *Iris* micropropagation**

Component	Concentration	
	Stock solution (mg/10 mL)	Volume of stock per 1 L of final medium (mL)
Organics		
Nicotinic acid	50	1
Panhotenic acid	100	1
Vitamin B <sub>1</sub>	20	1
Vitamin B <sub>6</sub>	10	1
Growth regulators		
2,4-D	10	10.0; 1.0 or 0.1
NAA	10	0.1 or 0.5
BA	10	0.3 or 1.0
KIN	10	2.5; 1.0 or 0.1
TDZ	10	1.0

- Sucrose, myo-inositol and agar powder, or phytigel as listed in Table 3.
- Detailed media formulations for all phases of micropropagation are given in Table 4.
- 1 N KOH or NaOH or 0.1 N HCl for pH adjustment.

**Table 3**  
**Composition of basal medium (BM) for *Iris* micropropagation**

Component	Concentration per 1 L
MS mineral solution	1×
Organics	1×
Sucrose	20–50 g
Myo-inositol	100 mg
Agar/phytagel	7/3 g

**Table 4**  
**List of media used for *Iris* spp. micropropagation**

Culture type	Phase	Composition for 1 L (plant regulators and additives in mg/L)	
Somatic embryogenesis	Induction	BM	1×
		Proline	250
		Casein hydrolysate	250
		2,4-D	10.0 or 1.0
	Maintenance	KIN	1.0
		BM	1×
		Proline	250
		Casein hydrolysate	250
Cell suspensions	Maintenance	2,4-D	1.0
		KIN	0.1
		BM	1×
		Proline	250
	Regeneration	Casein hydrolysate	250
		NAA	0.1
		BA	0.3 or 1.0
		or KIN	2.5
Shoot organogenesis	Induction	BM	1×
		Adenine sulfate	80
		Tyrosine	100
		TDZ	1.0
	Maintenance	BM	1×
		Adenine sulfate	80
		Tyrosine	100
		NAA	0.1
Germination/ rooting	Induction	BA	1.0
		BM	1×
		Adenine sulfate	80
		Tyrosine	100

### **2.5. Acclimation of Regenerated Plants to Ex Vitro Condition**

1. Peat, perlite, and sandy loam.
2. Growth containers.
3. Greenhouse.
4. Mist or humidity bench system.

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## **3. Methods**

This protocol describes micropropagation of irises using zygotic embryos and leaf bases as starting material. Successful plant regeneration has been accomplished via both somatic embryogenesis and organogenesis, depending on the type and concentration of plant growth regulators used for the induction of morphogenesis *in vitro*. After successful establishment of aseptic cultures, the further steps of micropropagation process of irises are: (1) Induction and maintenance of embryogenic and/or organogenic calli; (2) Germination of somatic embryos and/or shoot induction and multiplication; (3) Rooting of shoots; and (4) Acclimation of plantlets. The most efficient regeneration of plants can be achieved by using cell suspension cultures established from friable calli formed during the induction and maintenance phases (1).

### **3.1. Media Preparation**

#### *3.1.1. Preparation of Stock Solutions*

1. Prepare MS stock solutions as listed in Table 1. Dissolve one by one in distilled water, and bring up to final volume by adding distilled water (see Note 2).
2. Prepare stock solution of nicotinic acid, pantothenic acid, vitamin B<sub>1</sub> and B<sub>6</sub>, by dissolving each 50, 100, 20, and 10 mg of each, respectively, in individual aliquots of 10 mL deionized water. Use 1 mL/L stock solutions (see Table 2).
3. Prepare stock solutions of 2,4-D and NAA by dissolving 10 mg of each in 1 mL 96% alcohol. Add distilled water to raise the final volume up to 10 mL.
4. Prepare stock solutions of BA and KIN by dissolving 10 mg in 1 mL 1 N HCl or NaOH. Add distilled water to raise the final volume up to 10 mL.
5. Prepare stock solution of TDZ by dissolving 10 mg in 1 mL 1 N KOH. Add distilled water to raise the final volume up to 10 mL.
6. Store all stock solutions at 4°C in a refrigerator.

#### *3.1.2. Media*

1. Basal medium (BM): Mix the required volume of stock solutions, sucrose, myo-inositol and agar (Table 3).
2. Embryogenic calli induction medium (see Note 3): Prepare BM containing 30–50 g/L sucrose, supplemented with casein hydrolisate, proline, and plant growth regulators 2,4-D and KIN (Table 4).

3. Embryogenic calli maintenance medium: Prepare embryogenic calli induction medium modified to contain 1.0 mg/L 2,4-D and 0.1 mg/L KIN.
4. Shoot induction medium: Prepare BM containing 20 g/L sucrose. Add 80 mg/L adenine sulfate, 100 mg/L tyrosine, and 1.0 mg/L TDZ.
5. Shoot multiplication medium: Prepare BM containing 20 g/L sucrose. Add 80 mg/L adenine sulfate, 100 mg/L tyrosine, 1.0 mg/L BA, and 0.1 mg/L NAA.
6. Cell suspension medium: Embryogenic calli maintenance medium devoid of agar.
7. Root induction medium: Prepare BM containing 20 g/L sucrose, 80 mg/L adenine sulfate, and 100 mg/L tyrosine (Table 4).
8. Adjust pH of all media to 5.8 with 1 N NaOH or 0.1 N HCl before autoclaving at 121°C for 20–25 min.
9. Disperse the media in appropriate culture vessels; 25 mL into Petri dishes or 50–70 mL into presterilized Magenta boxes or glass jars.
10. Store culture medium at 25°C for a few days or at 4°C for a prolonged storage.

### **3.2. Plant Material Surface Sterilization**

#### *3.2.1. Seeds*

1. Rinse seeds with tap water and a few drops of Tween 20 for 1 h (see Note 4).
2. Surface-sterilize seeds with a 20–30% aqueous solution of 4% sodium hypochlorite for 20 min. Rinse three times with sterile distilled water.
3. Sterilize instruments for explant preparation (forceps, scalpels, needles) by dipping them in 90% ethanol and flaming. Cool them before use.
4. In a sterile environment, put seeds on BM without plant regulators (Fig. 1a).
5. Seal Petri dishes with Parafilm and place cultures under light at 25°C for a few days (see Notes 5 and 6).

#### *3.2.2. Leaf Bases*

1. Excise the basal portions of shoots from the greenhouse-grown plants or from field collections.
2. Wash thoroughly with tap water for at least 1 h to remove soil and other superficial contaminants (see Notes 1 and 7).
3. Immerse leaf bases in 70% ethanol for 1 min.
4. Decant ethanol and immerse leaf bases in 1% sodium hypochlorite containing Tween 20 (2–3 drops in 100 mL).
5. Rinse three times with sterile water.

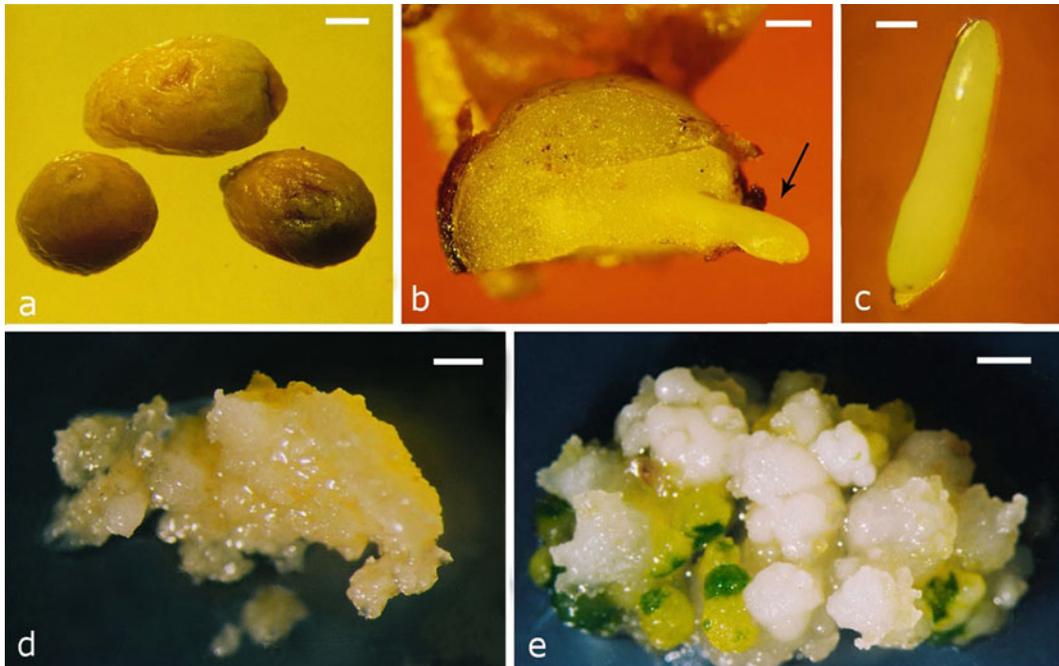


Fig. 1. Induction of morphogenesis in the culture of zygotic embryos of *Iris* sp. (a) Sterilized seeds of *Iris reichenbachii* after 2 days on growth regulators free medium. Bar 1.25 mm; (b) Isolation of zygotic embryos (arrow). Bar 0.5 mm; (c) Isolated zygotic embryo of *Iris pumila* placed on agar medium. Bar 0.5 mm; (d) Calli formed on *Iris sibirica* zygotic embryo, after 4 weeks of culture. Bar 1 mm; (e) Different types of calli formed on the same zygotic embryo of *Iris halophila* on media supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) (1.0 mg/L) and kinetin (KIN) (1.0 mg/L), after 8 weeks of culture. Bar 3 mm.

### 3.3. Callus Induction

#### 3.3.1. Zygotic Embryo Cultures

1. Sterilize instruments for explant preparation as in 3.2.1.3.
2. Isolate zygotic embryos from seeds by cutting seed coat longitudinally near the axis from left and right side (see Note 8). Make a very small cut at the micropilar region. Press the back side of the seed with forceps to push the zygotic embryo out (Fig. 1b).
3. Place the zygotic embryo on induction medium, seal Petri dish with Parafilm, and culture at 25°C for 4–6 weeks under light or in the dark (see Note 9) (Fig. 1c).
4. After 6–8 weeks, different types of calli should form (see Note 10) (Fig. 1d, e).

#### 3.3.2. Leaf Base Culture

1. Carefully separate basal portions of each leaf from the shoot and slice into ~5-mm thick pieces.
2. Place leaf bases on somatic embryogenesis induction medium supplemented with 1.0 mg/L 2,4-D and 0.1 mg/L KIN or media with 1.0 mg/L TDZ or 1.0 mg/L BA and 0.1 mg/L NAA for organogenesis induction (see Note 11).

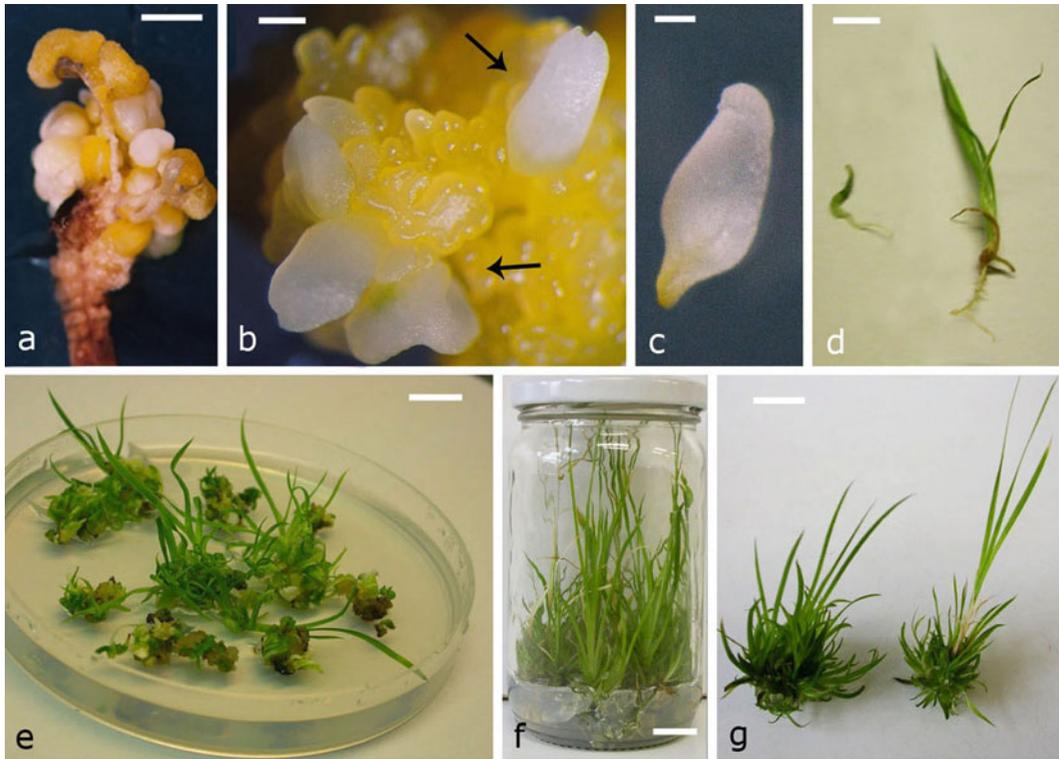


Fig. 2. Micropropagation of irises by leaf base culture. (a) Callus formation on leaf base of *I. sibirica* on MS medium supplemented with 2,4-D (10.0 mg/L) and KIN (1.0 mg/L) after 8 weeks. Bar 5 mm; (b) Embryogenic callus with somatic embryos (arrows) of *I. sibirica*. Bar 1 mm; (c) Somatic embryo of *I. reichenbachii*. Bar 1 mm; (d) Germinated somatic embryos of *I. sibirica* at different stages of development. Bar 10 mm; (e) Organogenic calli with shoots of *I. reichenbachii* formed on media supplemented with  $\alpha$ -naphthalenacetic acid (NAA) (0.1 mg/L) and 6-benzyladenine (BA) (1.0 mg/L). Bar 10 mm; (f) Shoot culture of *I. sibirica*. Bar 10 mm; (g) Axillary bud multiplication of *I. sibirica*. Bar 10 mm.

3. Culture for 6–8 weeks at 25°C in the dark to induce somatic embryogenesis (Fig. 2a) or under light for organogenic callus induction and shoot development (Fig. 2e).

### 3.4. Callus Maintenance

1. Transfer white or white-yellow calli to somatic embryogenesis maintenance medium (Table 4) and subculture every 4 weeks until well-developed white somatic embryos are formed (Fig. 2b).
2. Select green-white calli and transfer to shoot organogenesis medium supplemented with 0.1 mg/L NAA and 1.0 mg/L BA (Table 4; Figs. 1e and 2e).
3. After shoot induction, maintain cultures in glass jars by transferring 5–10 clumps per culture vessel every 4–6 weeks (Fig. 2f). Multiply shoots by axillary shoot formation without callus interphase (Fig. 2g).

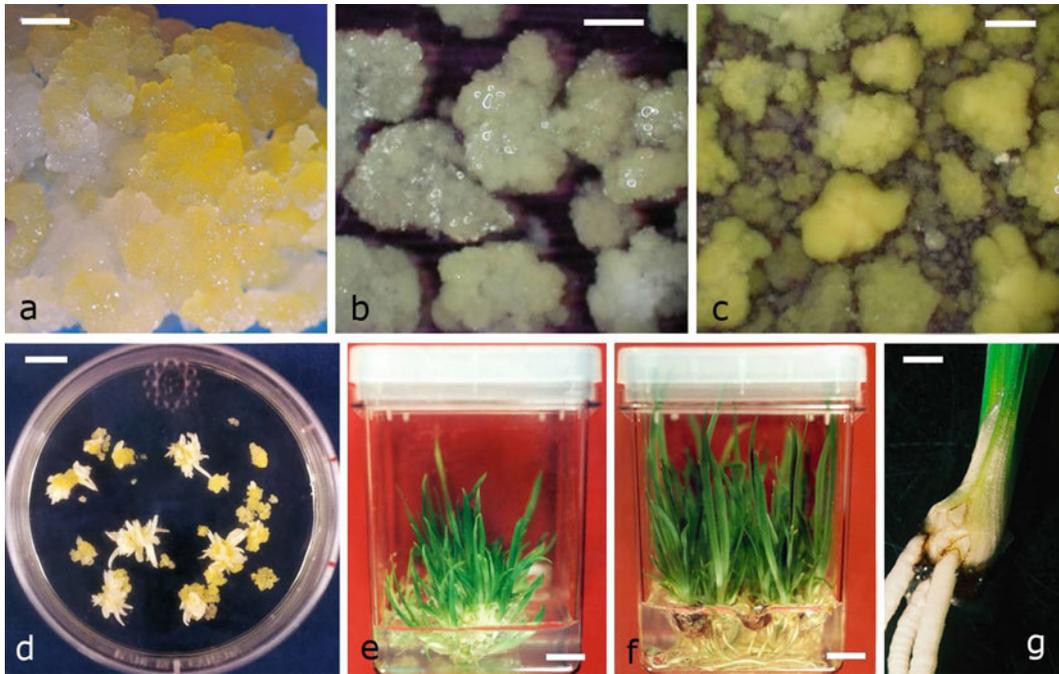


Fig. 3. Plant regeneration of irises by cell suspensions. (a) Friable embryogenic calli of *I. halophila* suitable for establishment of cell suspensions. Bar 1 mm; (b, c) Detail of *Iris germanica* cell suspension, cv. Hot Property (b) and cv. Asian Treasure (c). Bars 5 mm; (d) Callus clumps with shoot primordia formed on BM medium containing NAA (0.1 mg/L) and KIN (2.5 mg/L) after 4 weeks of culture in the dark. Bar 10 mm; (e) Development of shoots of *I. germanica* derived from cell suspensions on BM medium containing BA (0.3 mg/L). Bar 10 mm; (f) Rooted *I. germanica* shoots on BM growth regulator-free medium. Bar 10 mm; (g) Detail of rooted *I. halophila* shoot. Bar 10 mm.

### 3.5. Cell Suspension Cultures

1. To establish cell suspension, collect actively growing yellow or yellow-white calli from the solid cultures (Fig. 3a) and transfer at least 1 g into 250 mL flasks containing 50–75 mL cell suspension medium supplemented with 2,4-D and KIN (Table 4; Fig. 3b, c).
2. Maintain cell suspensions on a rotary shaker (60–100 rpm) at 25°C in the dark. Subculture every 3–4-weeks by transferring 10 mL suspension into fresh medium (see Note 12).
3. When cell suspension cultures are established (cell clumps 2–3 mm in size), pipette out liquid medium and spread cell clumps onto the solid shoot induction medium in Petri dishes.
4. Alternatively, pass 4-6 week-old suspension cultures through a 30-mesh stainless steel screen to remove large cell aggregates and collect the pass-through in 50 mL centrifuge tubes. Centrifuge at  $1,000 \times g$  for 5 min and resuspend pellet in a liquid shoot induction medium and inoculate on solid shoot induction medium in Petri dishes (Table 4).
5. Incubate for at least 5 weeks in the dark (Fig. 3d).

6. Subculture clumps of induced structures onto fresh medium and incubate at 25°C under light condition for 6 weeks to allow shoot development and elongation (see Notes 13 and 14) (Fig. 3e).

**3.6. Germination of Somatic Embryos**

1. Separate individual somatic embryos (Fig. 2c) from embryogenic calli and place them on solid BM in Petri dishes and culture for 4–6 weeks until plantlets are well developed (see Note 15) (Fig. 2d).

**3.7. Shoot Rooting**

1. Excise 5–8 cm long shoots from shoot cultures and transfer them to tubes, flasks, or Magenta boxes filled with the rooting medium (Table 4; Fig. 2f) (see Note 16).

**3.8. Acclimation of Regenerated Shoots to Ex Vitro Conditions**

1. Remove the developed plantlets from the vessel after 4–6 weeks and wash them gently with tap water to remove the adhering agar (Fig. 2g) (see Note 17).
2. Mix peat and perlite (3:1) or peat moss, perlite and sandy loam (1:1:1) and fill growth containers.
3. Plant plantlets (see Note 18) and put them on a mist bench, relative humidity 95–98%, in a greenhouse for 1–2 weeks (see Note 19).
4. After 1 year acclimation in the greenhouse (Fig. 4a, b), some plants may flower (Fig. 4c, d) and can be transferred to the field (Fig. 4e).



Fig. 4. Examples of the micropropagated *Iris* plants, grown in the green house or in the field. (a) Plants of several *Iris* species micropropagated by somatic embryogenesis. Bar 10 cm; (b) *I. germanica* plants regenerated from the cell suspensions. Bar 10 cm; (c–e) Flowering of in vitro regenerated plants of *I. reichenbachii* (c), *I. pumila* (d) and *I. sibirica* (e), Bars 2 cm, 2 cm and 10 cm, respectively.

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## 4. Notes

1. If in vitro-grown shoots are used as a source of explants, there is no need for sterilization of plant material (14).
2. Stock solutions grouped as MS major and minor inorganic nutrients and iron source can be used for preparation of BM. Commercially available MS solutions or powder mix can be used as well.
3. Improved induction of morphogenesis in vitro for some *Iris* species or cultivars can be achieved by addition of another auxin in the induction medium, such as 1.0 mg/L NAA (11, 15).
4. When seeds are highly contaminated by bacteria and fungi, leave them (in a beaker covered with gauze held in place by a rubber band) under running tap water for 1 day in order to soften the seed coat. Peel the seed coat and resterilize.
5. To avoid losing starting explants due to contamination, it is recommended to pre-culture sterilized seeds individually in test tubes filled with 5 mL basal hormone-free medium for 1–2 days.
6. Seeds of some *Iris* species like *Iris sibirica* germinate easily after seed coat removal.
7. To minimize contamination when using explants from the field- or greenhouse-grown shoots, wash shoots with tap water to remove soil and superficial contaminants than wash with antimicrobial hand soap solution. Rinse shoots thoroughly with tap water until the soap is completely removed and continue with the sterilization process as described earlier.
8. This procedure is very useful for round *Iris* seeds. Modify procedure for flattened seeds of other *Iris* species.
9. Induction of morphogenesis process can be achieved under light or in the dark, however, induction in the dark is better and faster.
10. During the induction period, three types of calli may be induced. Usually they are white (embryogenic) or yellow and green-white (organogenic). It depends on the species, genotype, media composition, and light conditions (8, 16, 17). One explant can often produce all three types of calli (see Fig. 2e).
11. TDZ can be used for the induction of shoot meristems in *Iris* cultures; regenerated shoots are short, thereby it is useful only for a short time during early growth (18). Continue shoot multiplication in BM containing 0.1 mg/L NAA and 1.0 mg/L BA.
12. The friable callus is most desirable for the establishment of fine cell suspension cultures (12, 16, 17, 19). *Iris* cell suspension

cultures are maintained by pipetting out the old medium and splitting the remaining callus tissue into two new flasks every 2–4-week interval (12, 17).

13. The regeneration of *Iris* plants from cell clumps of cell suspensions, via somatic embryogenesis and organogenesis, could be observed occurring concomitantly. It is often hard to conclude which process is dominant (12, 20).
14. Cell suspension cultures of irises maintained for a different period of time (2–5 years) usually retain high morphogenic potential and poor frequency of abnormal phenotypes (12, 17, 19).
15. Somatic embryos germinate successfully on BM plant-growth regulator free medium. Depending on the plant species, it is expected that 60–70% somatic embryos will germinate.
16. The rooting of *Iris* shoots is done successfully on BM containing full MS mineral solution. For some species or cultivars, a medium with half strength MS salts or BM medium supplemented with 0.1 mg/L indole-3-acetic acid (IAA) or NAA improves rooting.
17. The plantlets obtained by germination of somatic embryos (see Fig. 2d) can be very weak and delicate, contrasted with plantlets obtained by organogenesis and rooted on hormone-free medium, which are very strong with well developed root systems (see Fig 3g).
18. In order to ensure better acclimation, before transferring plants to the greenhouse, place micropropagated *Iris* plantlets into open glass vessels filled with tap water such that the roots are completely submerged for a few days.
19. Acclimation of regenerated plants (~100%) after transfer to soil is achieved by covering plants with Magenta boxes or similar transparent glassware for 2–3 days in order to maintain high humidity. Gradually reduce humidity by slightly lifting the Magenta box above the soil as plants show new growth. It is important to transfer a small number of plants at one time (5–10); water thoroughly and immediately cover with Magenta boxes to prevent water loss and wilting of plantlets. Maintain water in trays with pots, 3–4 cm deep, at all times during the acclimation period.

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## Micropropagation of *Gerbera* (*Gerbera jamesonii* Bolus)

Ghani Minerva and Surinder Kumar

### Abstract

*Gerbera* (*Gerbera jamesonii* Bolus) is one of the most popular ornamental flowers worldwide and used both as cut flower and potted plant. Some of them show excellent agronomic characters such as color, floral diameter, stem length, and vigor, which make this plant of commercial importance. Conventionally, multiplication is done through seeds or rhizome cuttings. Rapid multiplication of elite cultivars of *Gerbera*, with improved agronomic traits, has been achieved by using both direct and indirect tissue culture methods. Direct shoot regeneration was accomplished from stem apices on MS medium supplemented with 1 mg/L 6-benzyladenine (BA) and 1 mg/L kinetin. Indirect shoot induction succeeded from callus differentiation has been achieved on MS medium containing 2 mg/L 2,4-dichlorophenoxyacetic acid, 0.5 mg/L indole-3-acetic acid, and 2 mg/L BA. The in vitro shoots, 4–5 cm long, were rooted by quick dipping the shoot bases for 3–5 s in 2,000 mg/L indole-3-butyric acid solution followed by transfer to the pots containing farmyard manure, soil, and sand (1:1:1 by volume). Initially, in vitro plantlets were covered with glass jars to maintain a high relative humidity (85–90%). As soon as new shoot growth begins, relative humidity is decreased by exposing them to the open environmental conditions prior transferring to the glasshouse. Indirect shoot regeneration increased the frequency of somaclonal variations. The selected somaclones were used in developing new and novel cultivars.

**Key words:** BA, Callus, *Gerbera*, Mass propagation, Micropropagation, Ornamental plant, Regeneration, Shoot, Rooting

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### 1. Introduction

*Gerbera* (*Gerbera jamesonii* Bolus), a beautiful daisy, belongs to family compositae. Out of 40 known species, only *G. jamesonii* is cultivated. It is a native of South Africa and Asia, and has acquired commercial importance in recent years. This plant is named in the honor of German naturalist Traugott Gerber, and known as Transvaal or Barbeton daisy. Some of them show excellent agronomic characters such as flower color, floral diameter, vigor, and stem length. This plant is widely commercially produced by the floral industry both as cut flower and potted plant. The flowers are

hardy and can withstand vigorous transportation. They have long-keeping quality and fetch high market price. *Gerbera* has a wide range of attractive flower colors such as red, pink, orange, peach, maroon etc., which makes it a valuable ornamental species; it stands among the top ten cut flowers of the world (1).

Gerberas can be propagated by both sexual and asexual methods. Among the vegetative methods, multiplication through division of clumps has been used for several decades. These methods are however, too slow to be used commercially. Large-scale commercial production of *Gerbera* requires an easier, quicker, and economically viable method of propagation. Micropropagation method has been successful for rapid, large-scale multiplication of gerberas (2, 3). Tissue culture allows the production of disease-free plants which are free from seasonal variations and can produce over one million plants per year of an elite variety. Micropropagation of *Gerbera* is being used in many countries from a range of explants. Direct shoot regeneration using shoot tips as initial explant is most convenient method for mass propagation of *Gerbera* (4–6); however, indirect shoot regeneration has been achieved from calli derived from different explants such as leaf, petals, and floral buds (7–9). Adventitious shoot regeneration from flower buds/capitula is another favored method, followed by many researchers (10–13).

Detailed protocols for direct as well as indirect micropropagation of *Gerbera*, comprising culture establishment, rooting, and subsequent hardening and field establishment, are described.

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## 2. Materials

### 2.1. Surface Sterilization of Source Material

1. Tap water.
2. Autoclaved double distilled water.
3. Detergents (Teepol, Tween-20).
4. Fungicides (Carbendazim + Mancozeb, Bavistin; Indofil Chemical Co., Bombay, India), 0.2% (w/v).
5. Mercuric chloride (0.1%, w/v) or sodium hypochlorite (5%, v/v).
6. Magnetic bars.
7. 250 mL beaker (autoclaved).
8. Tissue culture facilities—Instruments (scalpels, blades, forceps, spirit lamps), laminar flow bench, magnetic stirrer, culture room.
9. Potted plants, 1-year old, growing in the glasshouse at  $25 \pm 2^\circ\text{C}$  under 16 h photoperiod. They are used as a source of explants.

**Table 1**  
**Culture media based on the formulation of MS (14)**

Component	Concentration (mg/L)
$\text{NH}_4\text{NO}_3$	1,650
$\text{KNO}_3$	1,900
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	440
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	370
$\text{KH}_2\text{PO}_4$	170
$\text{Na}_2\text{EDTA}$	37
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	27
$\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$	22
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	8.6
$\text{H}_3\text{BO}_3$	6.2
KI	0.830
$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	0.250
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.025
$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	0.025
Thiamine-HCl	0.1
Nicotinamide	0.5
Pyridoxine-HCl	0.5
Glycine	2.0
Myo-inositol	100
Sucrose	30,000
Agar	8,000

pH: 5.8

## 2.2. Culture Media

1. Four formulations of Murashige and Skoog (MS, (14)) media (see Table 1) are used for (a) shoot regeneration from shoot apice explants (b) callus induction from petal explants (c) shoot regeneration from callus, and (d) rooting of regenerated shoots.
2. Culture flasks (100 mL capacity, Borosil, Bombay, India).
3. Culture tubes (15 × 150 mm, Borosil, Bombay, India).
4. Non-absorbent sterilized cotton (for making plugs).

### **2.3. Acclimatization of Regenerated Plants to Ex Vitro Conditions**

1. Tap water.
2. Plastic pots (10 cm diameter).
3. Farmyard manure, soil, sand (1:1:1 by volume), or cocopeat (180 g/10 cm plastic pot).
4. Transparent plastic bags (250×150 mm) or glass jars (120×60 mm).

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## **3. Methods**

The micropropagation of *Gerbera* has been achieved by direct organogenesis from the cultured explants without callusing to avoid variation in the cultures, and by indirect (via callus) methods to induce variations. The surface sterilization of ex vitro explants is crucial and requires utmost care to ensure establishment of healthy cultures (see Note 1).

The core micropropagation protocol can be divided into three broad categories. Firstly, selection and establishment of source plants in the glasshouse to ensure the regular availability of explants. Secondly, in vitro establishment of shoot and callus cultures by using suitable concentrations of auxins and cytokinins. Shoots are directly formed and multiplied; induced callus is differentiated in shoot regeneration. Thirdly, root induction followed by period of acclimatization of in vitro plants to ex vitro conditions with gradual reduction in humidity, coupled with reduction in transpiration (15–19).

### **3.1. Preparation and Sterilization of Culture Media**

In general, high concentration of cytokinin is added in the culture medium for direct shoot regeneration, high auxin concentration is used for callus induction and rooting.

1. Prepare stock solutions of micro- and macro-elements and refrigerate. However, the stock solutions of various plant growth regulators should be prepared fresh at the time of use (see Note 2).
2. Thaw refrigerated stock solutions at room temperature.
3. Add other components such as sucrose and plant growth regulators as required.
4. Add appropriate volume of plant growth regulators from the stock solution of 1 mg/L to give required final concentration.
5. Prepare separate media for (a) direct shoot regeneration (b) callus production (c) shoot regeneration from callus according to the formulation given in Table 1.
6. For direct shoot regeneration from shoot apex, prepare medium containing 1 mg/L kinetin and 1 mg/L BA.

7. For callus induction from petal explants, amend medium with the addition of 2 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D).
8. Prepare medium to induce shoot differentiation on petal-derived callus by adding 2 mg/L BA and 0.5 mg/L indole-3-acetic acid (IAA).
9. Add 30 g/L (w/v) sucrose to the medium.
10. Adjust pH of the medium to 5.8 using 1 N HCl or 1 N NaOH solution.
11. Add 0.8% agar (w/v) (Difco-Bacto agar; Loba Chemie, Bombay, India) and dissolve it by boiling.
12. Dispense 25–30 mL medium in 100 mL flasks or 10–15 mL in culture tubes. Plug the flasks or culture tubes with non-absorbent cotton plugs, followed by sterilization in autoclave at 121°C temperature, 15 lb/in<sup>2</sup> steam pressure of 20 min.
13. Store the autoclaved media at room temperature in the dark for 1 week to ensure it is free from contamination (see Note 3).

### **3.2. Plant Source Material and Surface Sterilization**

1. Procure the source material from the certified commercial nurseries. Grow them in pots filled with farmyard manure, soil, sand (1:1:1 by volume) in the glasshouse till flowering by regular watering. The sterilization regime should be carried out to establish healthy cultures.
2. Excise shoot apices (0.5–1 cm) and petal explants (2–3 mm) from the mother potted plants (see Note 4).
3. Remove soil and dirt from the explants by washing for 1–2 h under gentle flow of running tap water.
4. Transfer explants to teepol solution (2%, v/v) or Tween-20 and wash with water by vigorous shaking for 30 min.
5. Treat explants with 0.2% fungicide (Carbendazim + Mancozeb solution; 0.2% w/v) prepared by dissolving 200 mg fungicides in 100 mL distilled water for 10–15 min (see Note 5). Keep stirring by using magnetic stirrer. Wash explants thoroughly under running tap water to remove any traces of fungicide from the explants.
6. Treat explants with 0.1% HgCl<sub>2</sub> solution for 2–3 min in laminar hood (see Notes 6 and 7), and keep shaking. Wash thoroughly with autoclaved double distilled water three to four times in order to remove toxic sterilants (see Note 8).
7. Soak excessive water from explants with autoclaved filter papers.

### **3.3. Culture and Maintenance of Explants**

Different protocols are adopted for direct (Fig. 1) and indirect (Fig. 2) shoot regeneration. Autoclaved instruments are used to carry out aseptic manipulations in laminar flow bench.

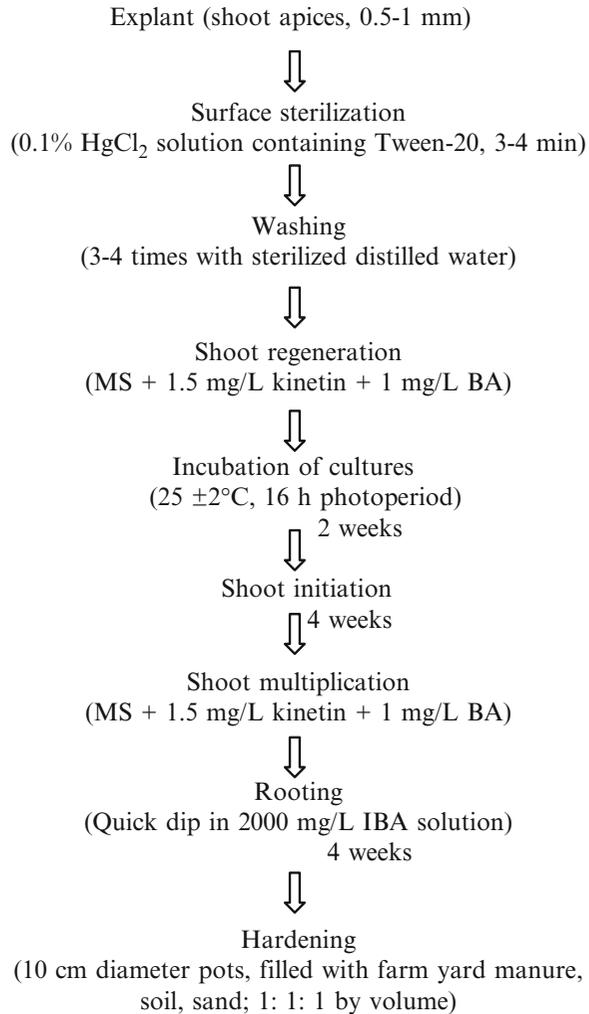


Fig. 1. Flow chart showing various steps to produce plants from shoot apices.

### 3.3.1. For Direct Shoot Regeneration

1. Use stem apices (0.5–1 cm) as initial explants (see Note 9).
2. Place one stem apices per culture tube or 3–4 apices in a culture flask, containing MS medium amended with 1 mg/L BA+1 mg/L kinetin for direct shoot regeneration.
3. Incubate shoot cultures at 25 ±2°C, 16 h photoperiod (50–60 μmol/m<sup>2</sup>/s, White cool fluorescent lamp; 40 W, Philips, India).
4. Shoot apices will grow in 2 weeks (Fig. 3a) and become 2 cm long in 4 weeks (Fig. 3b).
5. When shoots are approximately 2 cm long, separate and multiply them on the same medium (Fig. 3c).
6. Use 4–5 cm long shoots for root formation.

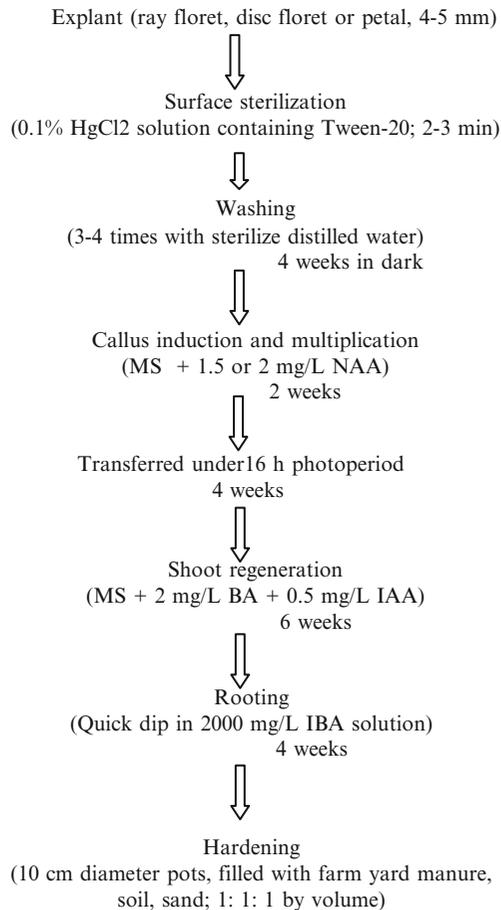


Fig. 2. Flow chart showing various steps to produce plants from ray florets, disc florets, and petals.

### 3.3.2. For Indirect Shoot Regeneration

1. Use petals for induction of callus.
2. Cut explants into small sections (2–3 mm) and place on the callus induction MS medium, containing 2 mg/L 2,4-D.
3. Incubate cultures in the dark to initiate callus and further growth (Fig. 4a).
4. Transfer 1-month-old calli, 4–6 mm in diameter, to plant growth chamber, and maintain under 16 h photoperiod, light intensity of 50–60  $\mu\text{mol}/\text{m}^2/\text{s}$ .
5. For shoot regeneration, transfer calli on shoot regeneration MS medium containing 2 mg/L BA and 0.5 mg/L IAA (see Notes 10 and 11).
6. Ideally shoot regeneration starts in 4–5 weeks (Fig. 4b).
7. Multiply regenerated shoots on the same medium. About 80% explants produced six shoots after 8 weeks (Fig. 4c, d).
8. Evaluate the percentage of explants producing callus, callus growth, and type and number of shoots per calli in each case.



Fig. 3. (a) Shoot regeneration from cultured shoot apex explants, (b) Adventitious shoot formation on explants after 4 weeks on MS medium containing 1 mg/L kinetin and 1 mg/L BA, (c) Shoot multiplication on MS medium containing 1 mg/L kinetin and 1 mg/L BA.

**3.4. Root Development on Regenerated Shoots In Vitro**

1. Select well developed, 4–5 cm long, shoots for root initiation (see Note 12).
2. Quick dip (3–5 s) basal portion of shoots in 2,000 mg/L indole-3-butyric acid (IBA) solution.
3. Some callus may form at the base of shoots prior to rooting. Root system develops in approximately 6 weeks.

**3.5. Acclimatization of Regenerated Plantlets to Ex Vitro Conditions**

When plants have developed a healthy root system, transfer them to the glasshouse for hardening. Separate shoots and quick dip the individual shoot in IBA solution (2,000 mg/L) (see Note 13).

1. Soak the rooted plantlets in fungicide solution (0.2% Carbendazim, w/v) for 1 h.

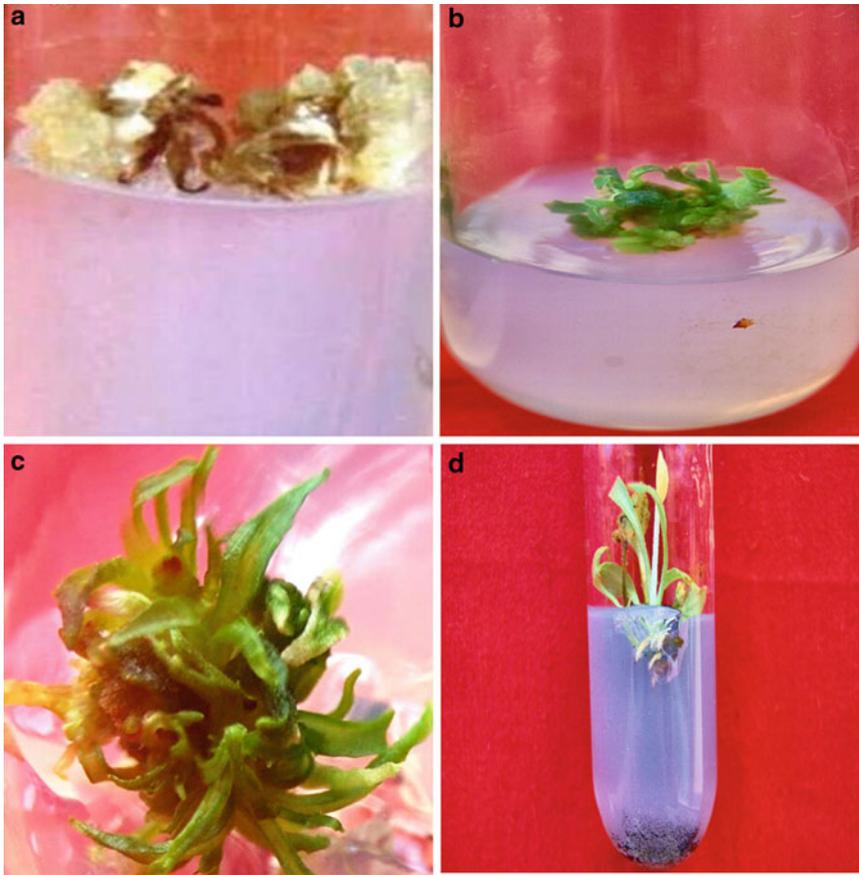


Fig. 4. (a) Callus formation from petal explants on MS medium supplemented with 2 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), (b) Shoot regeneration from callus on MS medium containing 2 mg/L BA and 0.5 mg/L indole-3-acetic acid (IAA), (c) Four week-old shoots, (d) Shoots after 8 weeks showing 5–6 leaves.

2. Transfer plantlets to the pots (10 cm diameter) containing farmyard manure, sand, and soil mixed in the ratio 1:1:1 by volume (see Notes 14 and 15).
3. Water the plants regularly, and cover them with inverted glass jars to maintain high relative humidity (85–90%) at  $25 \pm 2^\circ\text{C}$  (see Note 16).
4. Shoots are well established within 10–15 days. After 7 days remove jars for few hours per day to facilitate acclimatization of plants in ex vitro condition.
5. After 1 month, remove jars and transfer plants (4–5 cm long with 5–6 leaves) to the glasshouse and expose gradually to the ex vitro conditions (Fig. 5a–c).



Fig. 5. (a) Four week-old plants hardened in potting mixture containing farm yard manure, sand, and soil (1:1:1 by volume), (b) Hardened plants after 6 months, (c) Flowering plant of *Gerbera*.

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#### 4. Notes

1. Establishment of in vitro cultures of *Gerbera* can be difficult because of frequent contamination of initial explants.
2. Stock solutions of plant growth regulators should be prepared separately. Ensure that MS stocks are free from contamination at the time of use.
3. Media should be autoclaved properly and stored for 1 week prior to use, to ensure that it is free from microbial contamination.
4. Use young and juvenile plant parts because older parts develop wax layers and produce phenolic compounds.
5. Whenever surface fungicides fail to prevent contamination of cultures, use systemic fungicides (Carbendazim + Mancozeb).

6. Treatment time of explants with sterilizing agent should be monitored carefully. Too long or too short treatments of sterilization will result in browning or contamination of explants, respectively.
7. Ethanol, sodium oxychloride, and commercial bleach are effective sterilants.
8. Wash explants thoroughly during each step of surface sterilization to remove any traces of toxic sterilizing agent sticking to the explants. Gloves should be used while handling mercuric chloride solution.
9. Shoot regeneration from stem apices is highly genotype specific. The present protocol is effective for most of the local cultivars.
10. Dedifferentiation from the callus is a slow process and takes 3–4 months. Subculture callus to fresh culture medium every 4 weeks.
11. The best shoot regeneration from callus has been obtained with different concentrations of BA, however, the number of shoots per callus varied with the change in BA concentration.
12. IBA is most effective for root induction in *Gerbera*.
13. Remove dead or broken lateral roots prior to transfer to the potting mixture.
14. It is recommended to cut very long roots; 3–4 cm long roots are preferred for transferring to the soil.
15. Perlite, sphagnum, peat, sand, cocopeat, and farmyard manure, mixed in different ratios, are effective for ex vitro acclimatization of *Gerbera*.
16. It is mandatory to maintain 85–90% relative humidity around the plants during the first 10–15 days of transfer to pots.

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# **Part III**

## **Protocols for Micropropagation of Vegetables**

## Micropropagation of Onion (*Allium cepa* L.) from Immature Inflorescences

Pablo Marinangeli

### Abstract

In vitro plant production by direct organogenesis from immature flower heads is an ideal approach for clonal propagation of onions (*Allium cepa* L.). This technique ensures genetic stability, high propagation rate, and maintains donor plant of explants with an advantage over other means of in vitro regeneration. Onion micropropagation is usually applied in breeding programs, maintenance, and multiplication of cytoplasmic-male sterile lines for hybrid production, germplasm conservation, and as a tool for the application of other biotechnologies. For in vitro culture, mature onion bulbs are induced to reproductive phase by vernalization and forced to inflorescence initiation. Immature umbels are dissected from bulbs or cut directly when they appear from the pseudostem among the leaves. Disinfected inflorescences are cultivated in BDS basal medium supplemented with 30 g/L sucrose, 0.1 mg/L naphthalene acetic acid, 1 mg/L N<sup>6</sup>-benzyladenine, and 8 g/L agar, pH 5.5, under 16 h photoperiod white fluorescent light (PPD: 50–70 μmol/m<sup>2</sup>s) for 35 days. The regenerated shoot clumps are divided and subculture under the same conditions. For bulbification phase, the individual shoots are cultured in BDS basal medium containing 90 g/L sucrose, without plant growth regulators, pH 5.5, under 16 h photoperiod. Microbulbs can be directly cultivated ex vitro without acclimation.

**Key words:** *Allium cepa*, Immature inflorescence, In vitro bulbification, Micropropagation, Onion

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### 1. Introduction

A reliable in vitro culture protocol for direct regeneration of onion plants is a pre-requisite for micropropagation and application of other plant biotechniques. Several regeneration protocols were reported for *Allium* genus, specifically for *Allium cepa* L. (1) with variable success such as micro-shoot multiplication from axillary or adventitious buds (2, 3), and plant regeneration from floral organs at different developmental stages, namely inflorescences (4–6), and flowers (4, 7). In vitro plant regeneration potential is dependent on the genotype, culture medium composition, especially as for plant growth regulators (PGR), and environmental conditions (6, 8).

**Table 1**  
**MS (9) and BDS (10) (modified from Gamborg's**  
**B5 medium (14)) basal salt composition**  
**for the preparation of stock solutions**

Component	Concentration (mg/L)	
	MS	BDS
Macro (10× stock)		
CaCl <sub>2</sub> ·2H <sub>2</sub> O	3,322	1,500
KNO <sub>3</sub>	19,000	25,000
MgSO <sub>4</sub> ·7H <sub>2</sub> O	1,807	2,470
NaH <sub>2</sub> PO <sub>4</sub> ·2H <sub>2</sub> O	1,700	1,720
NH <sub>4</sub> NO <sub>3</sub>	16,500	3,200
NH <sub>4</sub> H <sub>2</sub> PO <sub>4</sub>	–	2,300
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	–	1,340
Micro (100× stock)		
CoCl <sub>2</sub> ·6H <sub>2</sub> O	2.5	2.5
CuSO <sub>4</sub> ·5H <sub>2</sub> O	2.5	2.5
H <sub>3</sub> BO <sub>3</sub>	620	300
KI	83	75
MnSO <sub>4</sub> ·H <sub>2</sub> O	1,690	900
NaMoO <sub>4</sub> ·2H <sub>2</sub> O	25	25
ZnSO <sub>4</sub> ·7H <sub>2</sub> O	860	200
Iron (100× stock)		
FeSO <sub>4</sub> ·7H <sub>2</sub> O	2,780	2,780
Na <sub>2</sub> EDTA	3,726	3,726

Most of the onion micropropagation protocols use Murashige and Skoog medium (MS; (9)) or BDS (10) basal salt medium (Table 1), supplemented with MS vitamins, sucrose as carbon source, and agar for solidifying the medium (1). PGRs commonly used for inducing shoot proliferation are 0.1–1 mg/L naphthalene acetic acid (NAA) and 1–10 mg/L *N*<sup>6</sup>-benzyladenine (BA; (6)).

When onions are produced by *in vitro* bulbification, the rooting phase is not required. Microbulbs are formed from single shoots or shoot clusters cultured in hormone-free BDS medium, containing 90 g/L sucrose. These microbulbs do not need special acclimation conditions when transferred to *ex vitro* condition and quickly produce sturdy plants (11). The reproductive meristem at early stages of floral differentiation provides juvenile tissues which differentiate shoots from one or few cells. This provides an explant with a greater potential to clone efficiently a genotype while maintaining the donor plant. The latter is not possible when onion seeds or bulbs are used as explant sources. Shoots or bulblets differentiation from inflorescences has been observed spontaneously *in vivo*, as a response

to high storage temperatures after vernalization of bulbs, or by spraying water or a BA solution to the juvenile inflorescences, or by previous elimination of the floral buds (4, 12).

In vitro shoot proliferation by direct organogenesis from inflorescences has also been reported (4, 11). In the cv Valcatorce INTA, multiple shoots were produced from inflorescences at an early stage of development (i.e., with first flowers at the primordial stage) (6). In this approach, vernalized bulbs were cultivated under conditions of floral development (12). Then, umbel primordia of 1.2–2.0, 2.0–4.0, and 4.0–6.0 mm wide (Fig. 1a) were cultured in BDS medium containing different concentrations of NAA and BA. On average, 20 shoots per explant were obtained, although the wider range was 0–58 shoots per explant. The highest number of micro-shoots was obtained when umbels of large size were cultured on a culture medium supplemented with 1 mg/L NAA and BA, respectively (Fig. 1b). Histological analysis showed that the micro-shoots were originated from the umbel receptacle that still remained meristematic without callus formation (Fig. 1c). Simultaneously, a low number of floral primordia in an advanced state of differentiation were observed, which finally produced rudimentary flowers. This chapter describes a protocol for the micropropagation of onion (*A. cepa* L.) from immature inflorescences.

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## 2. Materials

### 2.1. Explant Materials and Facilities

1. Collect in summer dormant bulbs (35–50 mm diameter) of onions (*A. cepa* L.), grown in field during the first year of cultivation. Alternatively, after the second year of the cultivation cycle, the remaining bulbs from flowering plants can be collected during the summer season. Clean, label, and store at 8–10°C until the chilling requirements have been met (12, 13). From this time onwards, bulbs sprout and flower; prepare and use explants from the immature inflorescences. Store unutilized vernalized bulbs at 4°C until use (see Notes 1 and 2).
2. Commercial peat moss-based potting mix (see Note 3).
3. Rectangular plastic containers (approximately 60 × 40 × 30 cm) with holes at the bottom to be used as pots.
4. Greenhouse or growth chamber with long-day photoperiod and temperature control in the range 10–18°C (see Note 4).

### 2.2. Collection, Surface Sterilization, Tissue Culture Materials, and Facilities

1. Sharp knife, cutter, and scalpel with no 11 blade.
2. 20% bleach for sterilizing tools during bulbs plantation, cutting bulbs, and extracting inflorescences.
3. 100 mL beakers with 75 mL tap water.

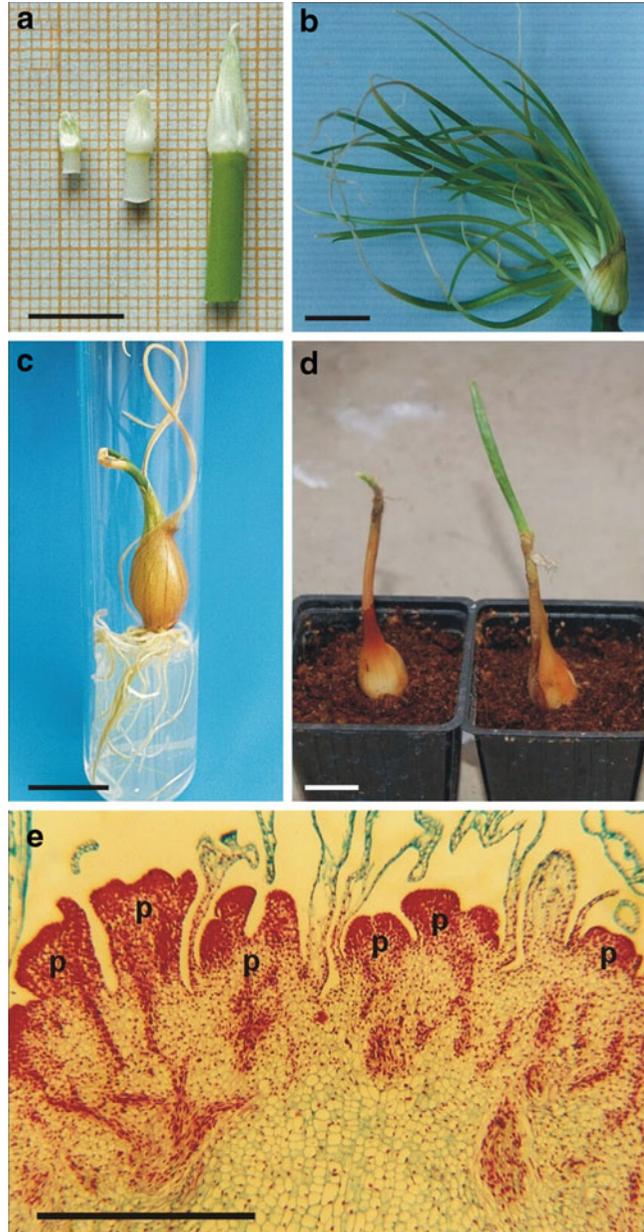


Fig. 1. Direct shoot proliferation and bulb formation from immature inflorescences (II). (a) II used as explants which were classified in three sizes according their diameter at the base: 1.2–2.0, 2.0–4.0, and 4.0–6.0 mm (bar, 10 mm). (b) Adventitious shoot formation on explants after 35 days culture in BDS medium with 1 mg/L NAA and 1 mg/L BAP (bar, 10 mm). (c) Microbulb developed after 45 days of shoot culture in BDS medium with 90 g/L sucrose (bar, 10 mm). (d) Microbulbs sprouting in greenhouse, 10 days after transfer to potting mix (bar, 10 mm). (e) Histological section of a 2–4 mm II, cultivated for 11 days in BDS medium with 0.01 mg/L NAA and 1 mg/L BAP. Shoot primordia (p) can be seen (bar, 1 mm).

**Table 2**  
**BDS-based onion proliferation and bulbification media**

Component	Component quantity to prepare 1 L of medium	
	Proliferation	Bulbification
BDS macro stock (mL)	100	100
BDS micro stock (mL)	10	10
BDS iron stock (mL)	10	10
Sucrose (g)	30	90
Thiamine (mg)	10	10
Nicotinic acid (mg)	1	1
Pyridoxine (mg)	1	1
Inositol (mg)	100	100
Naphthalene acetic acid (mg)	1	–
N <sup>6</sup> -Benzyladenine (mg)	1	–
pH	5.5	5.5
Agar (g)	8	8

4. Mesh cloth and rubber band.
5. Ethanol 70% in distilled or deionized water.
6. Commercial bleach solution (1.6% available chlorine), diluted with distilled or deionized water.
7. Tween 20.
8. Magnetic stirrer and stir bar.
9. Aluminum foil.
10. Sterile deionized or distilled water in bottles and 500-mL beaker for rinses.
11. Capped test tubes (25×150 mm) with 15 mL Onion Proliferation Medium (OPM; Table 2; see Note 5).
12. Capped test tubes (25×150 mm) with 15 mL of Onion Bulbification Medium (OBM; Table 2; see Note 5).
13. Aluminum foil packages containing five paper towels each, autoclave sterilized.
14. Tissue culture facilities and tools (laminar flow bench, scalpels, forceps, tool sterilizer, culture room).

### 2.3. Acclimation Materials

1. Sterile peat moss-based potting mix.
2. Plastic pots (diameter, 7 cm).

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## 3. Methods

Onion dormant bulbs can carry insects, mites, and saprophytic/pathogenic fungi and bacteria. Usually, these organisms are located between protective cataphylls, inside the neck or remaining roots and are invisible to the naked eyes. They grow together with the onion plant, reach the developing inflorescence, and subsequently contaminate *in vitro* cultures. Therefore, in order to avoid any further contamination, carefully examine the bulbs before cold storage, as well as before using to initiate *in vitro* cultures. Apply a phytosanitary treatment if necessary. Since 35–50 mm diameter onion bulbs usually produce only one inflorescence, use a number of bulbs equal to the number of explants required for micropropagation.

### 3.1. Plant Source Material

1. Fill the plastic box with substrate to  $\frac{3}{4}$  of its height.
2. Cut off 1 cm top part of the bulbs with a clean sharp knife. Disinfect frequently the knife by immersing the blade in 20% commercial bleach solution.
3. Plant bulbs 5–10 cm apart from each other, maintaining their apex-base polarity, half buried in the substrate.
4. Cultivate in the greenhouse at 10–18°C, preferably at long day photoperiod. Avoid temperatures over 24°C or sudden material transfer from cool to warm, e.g., from 14 to 27°C, because of inflorescence degeneration risk in competition with bulbification (see Note 1). Use tap water to water plants according to the requirement; fertilization and phytosanitary treatments usually are not necessary (see Note 6).
5. After 30–45 days, remove each plant individually from the container by holding the bulb and pulling straight up. If the roots are too moored to the substrate, use a hand shovel or a sharp tool to remove the plant without damaging bulb and pseudostem (see Notes 1 and 7).
6. Cut off pseudostem and roots with a clean sharp knife. Do not pull them to remove.
7. Extract the inner part of the bulb by preserving part of the basal plate and one or two cataphylls with the immature umbel inside. Proceed with care to dissect inflorescence with the floral stalk part; use scalpel to dissect by avoiding the inflorescence damage (Fig. 1a).
8. Place immature umbels into a clean 100-mL beaker filled with tap water.

### **3.2. Preparation and Sterilization of Culture Media**

Prepare OPM or OBM from the formulations in Tables 1 and 2 (see Note 5).

1. In an appropriate sized beaker, add distilled or deionized water up to  $\frac{1}{2}$  the final medium volume (i.e., 500 mL for 1,000 mL medium).
2. Add mineral salts from stocks, vitamins, sucrose, and growth regulators, stirring after each addition until dissolved.
3. Bring to final volume with distilled or deionized water, mix well, and adjust pH to 5.5 with NaOH or HCl 0.1 N.
4. Add agar, heat until gelling agent is fully dissolved, and dispense into autoclavable containers. Dispense 15 mL medium in each 25 × 150 mm tube. Cap tubes and place in autoclavable racks or in high-density autoclavable polyethylene bags.
5. Autoclave at 121°C for 20 min (118 kPa steam pressure).
6. Store the medium in a clean area and use within 2 weeks.

### **3.3. Surface Sterilization of Immature Inflorescences**

1. Cover the beaker containing immature inflorescences with mesh cloth, secure it with a rubber band, and place it under running tap water for 10 min.
2. Drain out water and remove the mesh cloth.
3. Immerse explants with 70% ethanol and allow standing for 1 min in a beaker.
4. Drain ethanol and immediately immerse explants in a bleach solution (1.6% available chlorine) in deionized water, containing two drops of Tween 20/L; stir the solution on a magnetic stirrer at low speed for 15 min.
5. Under a laminar flow hood, wash explants twice with sterile water for 2 min, and leave them in final rinse water.

### **3.4. Explant Preparation and Culture**

1. Excise an immature inflorescence with sterile forceps and place on a sterile paper towel, trimming the base of the floral stalk with a sterilized scalpel.
2. Place the explant inside the tube and maintain the physiological polarity by inserting the base of the floral scape in the medium. Cap the tube and seal with plastic wrapping film.
3. Place tubes in racks and transfer to the growth room at  $25 \pm 2^\circ\text{C}$ , under a 16 h photoperiod of white fluorescent light (PPD, 50–70  $\mu\text{mol}/\text{m}^2\text{s}$ ) for 35 days. Shoot clumps will appear from inside the explant by breaking the protective bract of the inflorescence.

### **3.5. Multiplication**

1. Divide shoot clumps into 3–4 smaller aggregates, trim leaves and roots, and move to fresh OPM at 3–4 week intervals.
2. Chop off longitudinally well developed individual shoots, in order to stimulate adventitious shoot proliferation.

3. Place tubes in racks and maintain in the growth room at  $25 \pm 2^\circ\text{C}$ , under the above mentioned light conditions, for 30 days.

### **3.6. Bulbification**

1. Allow shoots to individualize and grow. If necessary, subculture the proliferating clumps in hormone-free OPM medium for 2–4 weeks.
2. Trim leaves and place on OBM medium.
3. Allow microbulbs to grow up to 8–15 mm in diameter and external cataphylls to dry.
4. Roots and leaves can develop and leaves die when cataphylls dry (Fig. 1c).

### **3.7. Acclimation**

1. Remove microbulbs from tubes, cut green and dead leaves.
2. Remove remaining agar medium by rinsing microbulbs with running tap water.
3. Plant microbulbs in sterilized peat-moss potting mix and grow in the greenhouse (see Note 8). Normally microbulbs root and sprout immediately (Fig. 1d)

### **3.8. Cold Storage of Microbulbs**

1. Microbulbs can be stored at  $1\text{--}4^\circ\text{C}$  for up to 12 months without subculturing to fresh medium.
2. To grow cold-stored microbulbs, cut them longitudinally and culture in fresh OPM.
3. After adventitious shoot proliferation, proceed as described in Subheading 3.5.

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## **4. Notes**

1. Chilling requirements for flower induction, storage time duration, time for sprouting, and development of the inflorescence depend mainly on cultivar (12). This chapter describes long day onion “valenciana” type, cultivars Valcatorce INTA, Cobriza INTA, and Grano de oro. Working with other cultivars, culture conditions, and sampling time may require to be partially modified.
2. When bulbs are cold-stored for a longer time, bulbs sprout and cannot be used as explant source.
3. Commercial potting mix can be replaced by a substrate prepared mixing 1 or 2 parts of peat moss with 1 part of sandy soil.
4. When temperature rises beyond  $25^\circ\text{C}$  for a critical period of time during storage after vernalization, plants will not flower (12).

Similarly, temperature exceeding 25°C during sprouting and plant growth, inflorescence degenerates inside the bulb (12).

5. By replacing BDS basal salt mixture with MS salts (Table 1) results in lower growth.
6. Spray recommended pesticides and fungicides in contaminated plantings, paying attention to their composition to avoid hormonal effects. Avoid excessive use of nitrogen fertilizer, in order to prevent vegetative growth.
7. When immature inflorescences appear from the pseudostem among the leaves of donor plants, cut them directly. In this case there is more likelihood of contamination; moreover, some flower buds can develop during in vitro culture.
8. Microbulbs are hardy organs, like ordinary onion bulbs, and can be planted outdoors directly in the soil. Microbulbs can be dormant.

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## In Vitro Propagation of Cauliflower Using Curd Microexplants

Martin Kieffer and Michael P. Fuller

### Abstract

Cauliflower (*Brassica oleracea* var. *botrytis*) with its distinctive pre-inflorescence or curd is a remarkable member of the *Brassica* cabbage group. During curd development, intense and repetitive branching leads to a spectacular increase in size and the accumulation of millions of meristems at its surface. Although destined to produce flowers, most of these meristems are capable of regenerating vegetative shoots in vitro, making curd fragments an excellent material for the micropropagation of cauliflower. Most reported methods using these tissues were devised for the production of small clones of vitroplants as the true potential of curd fragments remained highly underestimated. We describe a technique exploiting fully this abundance of meristems and optimized for the large-scale in vitro propagation of cauliflower. The curd surface is first mechanically disrupted to break up the meristem clusters and generate microexplants carrying 1–3 meristems. These microexplants are then cultured at high density 1:100 (v:v) (microexplants:medium) in liquid medium containing Kinetin and indole-3-butyric acid (IBA) and produce thousands of microshoots in 12 days. After selecting the best quality microshoots on a sucrose pad, they are transferred *en masse* to a rooting medium supplemented with IBA. Four weeks later, rooted microshoots are carefully acclimatized before transfer to the field. This semi-automated protocol is rapid, cost effective, and well adapted for the production of clones of several thousands of plants by a single worker in a short space of time.

**Key words:** *Brassica oleracea* var. *botrytis*, Cauliflower, Curd, Micropropagation, Microshoot

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### 1. Introduction

Cauliflower is available all year round due to the intensive cultivation of short-cycle “summer heading” varieties in Spain and Italy in addition to the long-cycle “autumn heading” and “winter heading” varieties, mostly cultivated in the Atlantic coastal regions of France (Brittany), Spain, and the United Kingdom (Cornwall). Europe is the second largest producer of cauliflower and broccoli in the world behind Asia, and the main exporter—574,000 tons in 2007 (1). Commercial seed companies offer farmers F1 hybrid varieties with increasing uniformity: crop maturity, curd quality

(size, compactness, color), resistance to pest/disease and adverse weather conditions, as well as, importantly, a wide range of maturity time in order to supply the market throughout the year. Because of the relatively long life-cycle of cauliflower, breeding new F1 varieties is time consuming and seed companies are using plant cell and tissue culture methods and molecular breeding to gain speed and efficiency. Micropropagation and maintenance in vitro of elite lines such as self-incompatible or sib-incompatible inbred parent lines of F1 hybrids have been adopted early by the industry and is of great importance in cauliflower breeding programmes. Cauliflower in vitro cultures can be established using a range of tissues for the production of shoots (2–8) and somatic embryos (9), but the material of choice by most is the cauliflower curd (10–15). Curd fragments have been used for cauliflower micropropagation for over 40 years (10, 11), but despite a large body of work, the full shoot regeneration potential of this tissue has only recently been demonstrated (16). The surface of a mature cauliflower curd is composed of up to ten million meristems (17, 18) many of which are able to produce shoots in vitro (19). The key steps to maximize shoot regeneration are to carefully adjust the number of meristems placed in culture to avoid adverse competition, to supply an optimal combination of appropriate growth regulators to ensure shoots are fully competent to develop, and to use an efficient liquid culture system to guarantee uniform nutrient supply. In this chapter, we describe a low cost, semi-automated protocol for the rapid mass propagation of cauliflower using curd microexplants.

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## 2. Materials

### 2.1. Microexplant Production

1. Fresh quality cauliflower curd.
2. Ethanol 70% (v:v).
3. Commercial bleach solution (sodium hypochlorite 0.6% active chlorine, un-thickened with detergent) diluted 1:10 (v:v) with tap water.
4. Sterile reverse-osmosis/distilled water (2 L aliquoted in five Schott bottles (500 mL) sterilized in a bench autoclave).
5. Filter sterilized W5 osmotic protective solution (1 L) (Table 1; (20))
6. Commercial blender with sterilized autoclave resistant container (Model 800, Waring® Commercial, UK).
7. Autoclaved stainless (steel or brass) precision sieves (100 mm in diameter, wire mesh with aperture size 600, 300, 200 µm) (Endecotts Ltd., London, UK).
8. Autoclaved, 1,000 mL PYREX beakers (100 mm in diameter).

**Table 1**  
**Culture media and protective solutions**

	S23 medium (mg/L)	W5 solution (mg/L)
CoCl <sub>2</sub> ·6H <sub>2</sub> O	0.025	0.0
CuSO <sub>4</sub> ·5H <sub>2</sub> O	0.025	0.0
Na <sub>2</sub> EDTA·2H <sub>2</sub> O	37.26	0.0
FeSO <sub>4</sub> ·7H <sub>2</sub> O	27.8	0.0
H <sub>3</sub> BO <sub>3</sub>	6.2	0.0
KI	0.83	0.0
MnSO <sub>4</sub> ·H <sub>2</sub> O	16.9	0.0
Na <sub>2</sub> MoO <sub>4</sub> ·2H <sub>2</sub> O	0.25	0.0
ZnSO <sub>4</sub> ·7H <sub>2</sub> O	8.6	0.0
CaCl <sub>2</sub>	332.2	13,875.0
KH <sub>2</sub> PO <sub>4</sub>	170.0	0.0
KNO <sub>3</sub>	1,900.0	0.0
MgSO <sub>4</sub>	181.0	0.0
NH <sub>4</sub> NO <sub>3</sub>	1,650.0	0.0
Glycine	2.0	0.0
NaH <sub>2</sub> PO <sub>4</sub>	170.0	0.0
NaCl	0.0	9,000.0
KCl	0.0	901.0
Thiamine HCl	0.5	0.0
Myo-inositol	100.0	0.0
Nicotinic acid	0.5	0.0
Pyridoxine HCl	0.5	0.0
Adenine sulphate	80.0	0.0
Sucrose	30,000.0	0.0
Agar	0.0 or 7,000.0	0.0
pH	5.8	5.8

Add to the microshoot culture medium the growth regulator: Kinetin at 2.0 mg/L and IBA at 1.0 mg/L and the antibiotics: Cefotaxime at 100 mg/L and Vancomycin at 100 mg/L. Add to the rooting medium the growth regulator: IBA at 2.0 mg/L

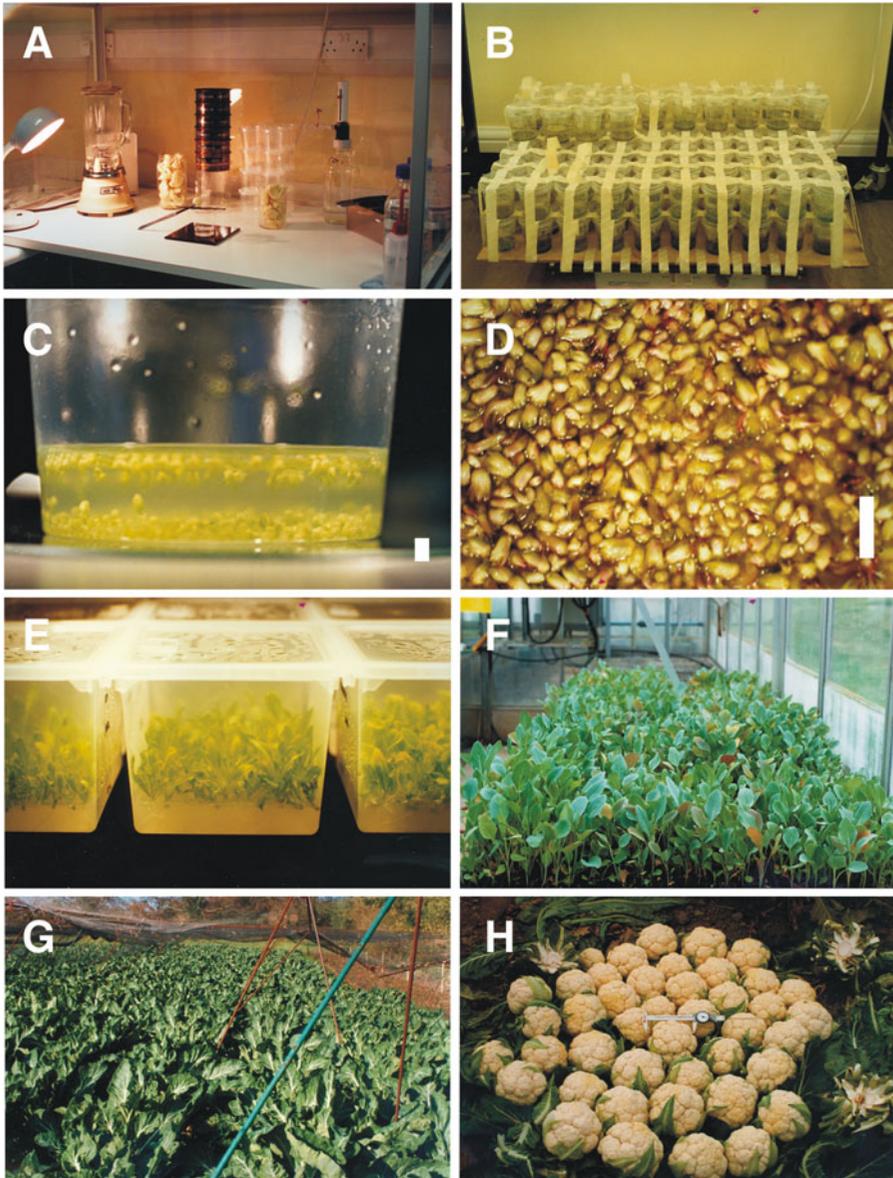


Fig. 1. Micropropagation of cauliflower using curd microexplants. (a, b) Experimental set up for the production and culture of curd microexplants. (c) Microshoot regeneration after 12 days of culture in agitated liquid culture medium containing 2.0 mg/L Kinetin and 1.0 mg/L indole-3-butyric acid (IBA). (d) Selected microshoots after the sucrose flotation step. (e) Developing microshoots after transfer on rooting medium containing 2.0 mg/L IBA. (f, g) Weaned vitroplants in the glass-house and in field trial. (h) Sample of harvested curds (bars=5 mm).

9. Tissue culture equipment: culture pots or jars, tweezers, scalpel, glass bead sterilizer, or spirit burner to sterilize instruments, laminar flow cabinet, bench autoclave, culture room (International Guidelines for Controlled Environments and Plant Tissue Culture) (Fig. 1a; (21, 22)).

## **2.2. Microexplant Culture**

1. S23 culture media derived from the formulation of Murashige and Skoog (MS) (23) modified by Anderson and Carstens (24) for microshoot production and rooting.
2. Small stainless volumetric measures (Smidgen, Endurance 3-pc, RSVP International Inc., USA).
3. Disposable sterile culture vessel with lids (Insulpak Ltd., UK or similar).
4. Orbital shaker with large platform.
5. Sucrose solution (0.5 M, 171 g/L) (w:v) filter sterilized using 0.22 µm syringe filter.
6. Nylon mesh (aperture size 200 µm) (Sefar Ltd., UK).
7. Sterile volumetric measuring cups (40 mL) (STIPlastics, France or similar).
8. Disposable large square “vitro vent” culture container (Duchefa Biochemie bv, Haarlem, NL or similar).

## **2.3. Acclimation of Vitro Plants**

1. Erin modular trays (Avoncrop Ltd., UK or similar).
2. Multipurpose compost (Mole Valley Ltd., UK or similar).
3. Plant culture facilities (glasshouse, humidifier, seed tray cover).
4. Fertilizer, Phostrogen® (N:P:K, 10:10:27) (Phostrogen Ltd., UK or similar).

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## **3. Methods**

The usual blueprint for plant micropropagation requires the establishment of virus-free shoots *in vitro* by shoot apical meristem culture and the long and labor intensive bulking up of plants through repetitive cycles of shoot fragmentation and nodal explant cultivation. With cauliflower, the *in vitro* large-scale culture of curd derived-meristems enables the rapid production of thousands of vitroplants in one cycle without sub-culturing.

### **3.1. Preparation of Culture Media**

1. Prepare culture media in large Schott Bottles using MS pre-mixed salts (4.4 g/L) (M5519, Sigma-Aldrich Ltd., UK) supplemented with Thiamine HCl (0.4 mg/L), Adenine Sulphate (80.0 mg/L), and sucrose (30 g/L) (Table 1). Add agar (7 g/L) (w:v) (Agar Bacteriological No 1, Oxoid Ltd., UK) to the rooting medium.
2. Adjust pH to 5.8 using 1 M HCl and 1 M NaOH.
3. Sterilize media by autoclaving at 121°C for 15 min. This also dissolves the agar.

4. In the laminar flow cabinet, add the required amount of the growth regulators Kinetin (2.0 mg/L) and indole-3-butyric acid (IBA) (1.0 mg/L) to the microexplant culture medium. Use concentrated stock solutions of growth regulators at 0.1 mg/ $\mu$ L (Kinetin is dissolved in 1 M NaOH, IBA in 70% ethanol). Add only IBA (2.0 mg/L) to the microshoot rooting medium.
5. To control microbial infection, the antibiotics Cefotaxime (100 mg/L) and Vancomycin (100 mg/L) are added to the microexplant culture medium. Antibiotics are weighed, dissolved in water, and filter sterilized using 0.22  $\mu$ m syringe filter before addition to the autoclaved medium. Alternatively the antibiotics can be successfully replaced by the addition of Plant Preservative Mixture (PPM<sup>TM</sup>, 1.0 mL/L) (Plant Cell Technology Inc., USA), although this may affect the growth of microshoots from some varieties.
6. Aliquot 20 mL microexplants culture medium to each culture vessels using a sterile bottle dispensing pump or a measuring cylinder.

### **3.2. Preparation of Curd Microexplants**

The curd outer meristematic layer is harvested and mechanically disrupted in a commercial homogenizer before grading through precision sieves to produce microexplants each carrying 1–3 meristems.

1. Using a scalpel, cut the cauliflower curd into large pieces (or florets) of 2–5 cm in diameter by sectioning the basal branches, taking care not to bruise the curd surface meristematic layer. Reduce the length of the branches as much as possible.
2. Surface sterilize florets in large jars by soaking them successively in 70% ethanol for 30 s, followed by 15 min in 10% commercial bleach sodium hypochlorite 0.6% active chlorine and sequentially washed for 5, 10, and 15 min in distilled sterile water (see Note 1).
3. To separate the upper meristematic layer from the mass of non-responsive branch tissues, hold florets with sterile tweezers and cut off the fine sliver of meristematic tissues (no more than 1 mm thick) with a sharp sterile scalpel (see Note 2). To avoid desiccation and osmotic damage, the shaved off meristem clusters are recovered in 50 mL W5 solution (or liquid MS medium).
4. To complete microexplant production, transfer meristem cluster solution into a commercial blender (Waring Model 800) and homogenize for 25 s at approximately 17,000 rpm (see Note 3). This strong mechanical treatment generates a very large amount of microexplants of various sizes including meristematic bearing pieces and tissue debris. The best explants are

the ones carrying 1–3 meristems with a size between 200 and 300  $\mu\text{m}$  in diameter (19) (see Note 4).

5. To recover these microexplants, the homogenate is graded into size-classes using precision sieves (600, 300, 200  $\mu\text{m}$ ) (Endecotts Ltd., UK). Stack the sieves in descending mesh aperture size and place them on a closely fitting (1,000 mL) beaker. After pouring the microexplant solution through the sieves, use 1 L sterile W5 solution (or liquid MS medium) to rinse the blender and pass through the sieves twice to facilitate grading (see Note 5). The explant fraction between 300 and 600  $\mu\text{m}$  can be homogenized once more and the solution passed again through the sieves. Microexplants recovered on the 200  $\mu\text{m}$  sieve are ready for culture (a medium size curd, gives on average 4–5 mL of microexplants).

### **3.3. Production of Microshoots**

To maximize nutrient supply and the growth of thousands of unrooted microshoots, microexplants are cultivated in liquid S23 medium supplemented with Kinetin (2.0 mg/L) and IBA (1.0 mg/L) at density of approximately 1:100 (v:v) (microexplants:medium) (see Note 6). The best quality microshoots are then selected using a density based screen before transfer to rooting medium.

1. For each container (Insulpak Ltd., UK) add 200  $\mu\text{L}$  microexplants (see Note 7) to 20 mL culture medium and after closing the containers fix them securely on an orbital shaker (Fig. 1b).
2. Incubate at 23°C under low light intensity, spectral photon fluence 50–100  $\mu\text{mol}/\text{m}^2/\text{s}$ , 16 h photoperiod, and constant agitation at 150 rpm for 12 days. Depending on the responsiveness of the cauliflower variety used, up to 300 un-rooted microshoots (1–2 mm long) are recovered per container (see Note 4) (Fig. 1c).
3. Dispose of containers with signs of bacterial or fungal contamination, autoclaving prior to disposal.
4. Select the best quality microshoots- small, dense celled, non-vitrified (Fig. 1d) by floating all developing explants on a sucrose pad. In the laminar flow cabinet, drain the culture medium and remove the microshoots using a sterile spatula before placing them in 30 mL sterile sucrose solution (0.5 M) (see Note 8). The best microshoots remain at the surface while the “vitrified” propagules and most debris sink to the bottom of the container. The quality microshoots can easily be scooped out of the solution using sterile tweezers holding a small piece of sterile nylon mesh (1.5 cm  $\times$  1.5 cm) (Sefar Ltd., UK) and forming a small net. Place the selected microshoots in fresh sterile culture medium to rinse off the sucrose solution before transfer to rooting medium.

### 3.4. Rooting Procedure

To induce the production of a root system, microshoots are transferred at a density of 100 propagules per large (10 cm × 10 cm) square “vitro vent” container (Duchefa biochemie by, Haarlem, NL) containing 85 mL semi-solid rooting medium, solidified with agar 7.0 g/L. Transferring each microshoot individually is very labor intensive but they can also be transferred in bulk using sterile kitchen measuring cups (STIPlastics, France) after suspending them in a viscous solution.

1. To obtain a medium of the right viscosity, transfer 500 mL semi-solid rooting medium (see Note 9) into a Waring blender and homogenize for 10 s.
2. Add the microshoots gradually to the viscous medium and mix them with a long sterile spatula until the density of approximately 100 microshoots per 40 mL medium.
3. Transfer 40 mL microshoot suspension using sterile volumetric cups onto each prepared “vitro vent” container on top of the semi-solid medium and spread the microshoots evenly by spinning the culture container gently.
4. Incubate the culture for 4 weeks at 23°C, spectral photon fluence 200 μmol/m<sup>2</sup>/s, 16 h photoperiod. IBA is highly responsive for inducing roots at 2.0 mg/L concentration, 3–6 roots are produced per cauliflower vitroplant (Fig. 1e).

### 3.5. Weaning (Acclimation) of Vitroplants

Rooted shoots are carefully acclimatized in the glasshouse before transfer to the field. Cauliflower vitroplants are delicate to wean due to their initial low level of leaf epicuticular wax and poor photosynthetic activity which render them sensitive to desiccation and intense light (25, 26). The following weaning approach minimizes vitroplants losses and maximizes the recovery of high quality plants.

1. Recover the rooted shoots from the culture containers and remove any remaining agar by washing the roots in water. Although the majority of the plants have one shoot, some explants can develop 2–3 shoots connected to the same root system. Cut off the extra shoots using a scalpel and transfer plants with only one shoot. The use of a fungicide dip can decrease the chances of damping off during weaning.
2. Transfer the plants in multipurpose compost in the individual modules of large plastic culture trays.
3. Maintain the plants for about 10 days at 20°C and high humidity in a propagator then place them on a mist bench with base heating at 23°C for another 10 days (see Note 10).
4. Transfer the plants to the glasshouse and fertilize them every week with a commercial nutrient solution such as Phostrogen™ (4 g/L) (N:P:K, 10:10:27) (see Note 11). Vigorous plants are ready to transfer to the field after 2–3 weeks of culture (Fig. 1f–h; see Note 12).

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## 4. Notes

1. Limit the risk of in vitro contamination by discarding before aseptization any florets with signs of damage or infection. In general, use curds still protected by wrapper leaves and, if possible, curds cultivated in a polytunnel or greenhouse with low exposure to rain water or overhead irrigation. Cauliflower curds stored in a cold room at 4°C can remain responsive for 2–3 weeks as long as they are not damaged.
2. Cut meristematic layer as thinly as possible to maximize explant production. The removal of the sub-tending stem tissues facilitates the ability of the meristems to develop into shoot and limits the induction of unwanted “neo formed” meristem (19).
3. Adjust empirically the duration of homogenization for each blender by assessing the amount of microshoot produced per volume of microexplants and the total amount of microexplant produced vs. the duration of treatment. Beware that longer treatment not only produces more small explants it also generates more debris (19).
4. This size-class is optimal for most cauliflower genotypes but “summer heading” varieties can be less responsive and may need bigger microexplants. If necessary, use a 400 µm mesh aperture sieve and recover the explants 200–400 µm in diameter.
5. Microexplants tend to clog the sieves and the solution can generate foam. To eliminate these problems place the sieves stack on a beaker equipped with a side nozzle and connect it to a small vacuum pump to generate a mild suction. To facilitate microexplant recovery on the last sieve, pour the W5 solution (or MS medium) from one direction to the next to gather the microexplants at one side of the mesh.
6. Microexplant concentration is optimized to ensure good nutrient supply but is maintained at a suboptimal level to limit “vitrification” associated with fast microexplant growth (16).
7. Measure the volume of microexplants placed in culture with commercially available measuring spoons (Smidgen, Endurance 3-pc, RSVP International Inc., USA).
8. The sucrose concentration is optimized for the efficient density separation of the good quality microshoots from the defective one but it is not recommended to leave them in the sucrose solution for a long period as it can damage them by osmotic shock. Bract (leaf like) fragments can be recovered from low quality curds and develop rapidly in culture. These unwanted explants are not separated on the sucrose pad but their large size makes them easy to remove with sterile tweezers.

9. Shake vigorously the bottle containing the semi-solid medium, to break up the gel and facilitate its transfer in the blender.
10. These are the optimal conditions for weaning but success can still be achieved without the mist bench.
11. Recovered vitroplants are variable in size but regular fertilizing with a standard fertilizer reduces this variability by the time of transfer to the field.
12. If needed, vitroplants can be stored at 4°C under low light intensity for several weeks before transfer to the field.

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## Micropropagation of *Asparagus* by In Vitro Shoot Culture

Nataša Štajner

### Abstract

*Asparagus officinalis* is most extensively studied species within the genus *Asparagus*, which is well known as garden asparagus. This species is dioecious with unisexual flowers, which means that generative propagation gives roughly equal number of male and female plants. Male plants are high yielders and preferred commercially over female plants. Tissue culture techniques could efficiently promote vegetative propagation of male plants and pave the way for efficient plant breeding. This chapter describes an efficient micropropagation protocol for developing rapid growing in vitro *Asparagus* shoot cultures. The source of explants, inoculation, and shoot proliferation, followed by shoot propagation, rooting, and acclimatization is described. The optimal medium for *Asparagus* micropropagation described in this chapter is composed of MS macro- and microelements and a combination of auxins and cytokinins. Plant growth regulators NAA, kinetin, and BA were used in various concentrations. Three different media representing the whole micropropagation protocol of *Asparagus* are described; medium for shoot initiation, medium for shoot multiplication, and medium for root formation. By in vitro propagation of *Asparagus*, root initiation is difficult, but can be promoted by adding growth retardant ancymidol which also greatly promotes shoot development and suppresses callus formation.

**Key words:** Acclimatization, Ancymidol, *Asparagus officinalis*, Micropropagation, Shoot initiation, Rooting

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### 1. Introduction

The genus *Asparagus* consists of nearly 200 species, which are found as herbaceous perennials, tender woody shrubs, and vines (1); and is classified into three subgenera: *Asparagus*, *Protasparagus*, and *Myrsiphyllum* (2). The species of the first subgenera are dioecious with unisexual flowers, whereas the remaining two subgenera include only hermaphroditic plants. They are grown worldwide but mainly originated from Asia, Africa, and Europe. The origin of *Asparagus officinalis* is believed to be the eastern Mediterranean

region (3). Some species have economic value such as ornamentals (*Asparagus plumosus*, *A. densiflorus*, *A. virgatus*, *A. myriocladus*, and *A. retrofractus*), and medicinal properties (*A. racemosus*, *A. verticillatus*, *A. adscendens*, and *A. curillus*). The wild *A. maritimus* and *A. acutifolius* are used in human diets. However, most economically important *Asparagus* species is garden asparagus (*A. officinalis*), which is a highly prized vegetable. The gene pool of *A. officinalis* is relatively limited, and attempts were made for introgression of agronomical important traits from wild relatives', such as disease resistance (4), salt tolerance from *A. maritimus*, drought tolerance from *A. acutifolius* or acidic tolerance from *A. tenuifolius* (5, 6). Previous attempts failed to develop viable interspecific hybrids with *A. officinalis* (6, 7).

Micropropagation of *A. officinalis* has been extensively studied (8–11). The optimal medium used for asparagus micropropagation is composed of Murashige and Skoog (MS; (12)) macro- and microelements and is supplemented with a combination of auxins and cytokinins. By in vitro propagation of *Asparagus*, root initiation is difficult, but can be promoted by adding growth retardant ancymidol (13), which also greatly promotes shoot development and suppresses callus formation (14). Ancymidol effect on shoot culture could be due to simultaneous disruption of apical dominance and alteration of the carbohydrate metabolism, as noted for *A. officinalis* (15).

The vegetative propagation of *Asparagus* is ideal to produce only male plants. They are commercially preferred due to higher yields than female plants. The generative propagation of *Asparagus* gives a roughly equal number of male and female plants. Before the introduction of tissue cultures, the only method of vegetative propagation was rhizome division which is not a cost-effective method and produces only a few genetically identical plants from a single plant. However, recently developed various tissue culture techniques have a great potential of rapid and efficient plant breeding.

This paper describes an efficient micropropagation protocol for developing rapid growing in vitro *Asparagus* shoot cultures. The source of explants, inoculation, and proliferation of shoots, followed by shoot propagation, rooting, and acclimatization is described.

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## 2. Materials

### 2.1. Plant Material

*Asparagus* plants were maintained in the greenhouse of Agronomical Plant Section, Biotechnical Faculty, University of Ljubljana. They can be grown in the field as well as in the greenhouse, preferably as potted plants. Five to ten centimeters long

young spears, emerging from the plants, are used as explants. The seeds of *Asparagus* can also be used as a source material and can be germinated directly on sterile filter paper.

1. Growth substrate—Klasmann Tonsubstrat (Klasmann-Deilmann GmbH, Germany) composed of white and frozen trough sphagnum peat, medium grade with clay to optimize pH and salt levels (electrical conductivity 40 mS/m, pH value 5.5–6.5, fertilizer 1.5 kg/m<sup>3</sup>). The substrate has a high absorptive capacity, enriched with water-soluble fertilizer, and is ideal for plants with delicate root system.
2. Plastic pots (25 cm in diameter).
3. Tap water.

### **2.2. Sterilization**

1. Ethanol (70%).
2. Dichloro-isocyanuric acid (Sigma) and nonionic detergent Tween 20 (Sigma).
3. Autoclaved (sterile) reverse-osmosis water; 50 mL aliquots in a 100 mL Erlenmeyer flask, and 1,000 mL in screw-capped Duran glass bottles.
4. Autoclaved sieves and 1,000 mL beakers.
5. Sterilized instruments (forceps, scalpels).
6. Sterile glass Petri dishes.
7. Laminar air flow chamber.
8. Micro-incinerator (PBI International, Milano) for sterilization of instruments (see Note 1).

### **2.3. Tissue Culture Media**

1. Pipettes.
2. Plastic beaker.
3. Magnetic bar.
4. NaOH and HCl solution (0.1–1 M).
5. Vessels for plant tissue culture: glass baby jars (175 mL, 66 × 59 mm) or Magenta<sup>®</sup> boxes (95 × 65 mm).
6. Screw-capped Duran glass bottles, 1,000 mL capacity.
7. MS basal media (Table 1) with combination of different growth regulators for shoot initiation (Table 2), shoot multiplication (Table 3), and root formation (Table 4) of the tissue culture.
8. Tissue culture facilities: electronic scale, magnetic stirrer, autoclave, microwave, laminar flow chamber, plant culture room.

### **2.4. Acclimatization of Regenerated Plants**

1. Tap water.
2. Scissors.
3. Plastic pots (12 cm in diameter).
4. Potting substrate (Klasmann Tonsubstrat).

**Table 1**  
**MS basal medium (12)**

Component	Concentration
Sucrose (%)	3
Bacto-agar (Difco Laboratories, USA) (%)	0.7
Macro- and microelements (mg/L)	
NH <sub>4</sub> NO <sub>3</sub>	1,650.00
H <sub>3</sub> BO <sub>3</sub>	6.20
CaCl <sub>2</sub> ·2H <sub>2</sub> O	332.20
CoCl <sub>2</sub> ·6H <sub>2</sub> O	0.025
CuSO <sub>4</sub> ·5H <sub>2</sub> O	0.025
Na <sub>2</sub> EDTA·2H <sub>2</sub> O	37.26
FeSO <sub>4</sub> ·7H <sub>2</sub> O	27.80
MgSO <sub>4</sub> ·7H <sub>2</sub> O	180.70
MnSO <sub>4</sub> ·4H <sub>2</sub> O	16.90
KI	0.83
KNO <sub>3</sub>	1,800.00
KH <sub>2</sub> PO <sub>4</sub>	170.00
Na <sub>2</sub> MoO <sub>4</sub> ·2H <sub>2</sub> O	0.25
ZnSO <sub>4</sub> ·7H <sub>2</sub> O	8.60
Vitamins	
Myo-inositol	100
Glycin	2.00
Nicotinic acid	0.50
Thiamine-HCl	2.00
Pyridoxine-HCl	0.50

**Table 2**  
**Growth regulators for shoot initiation medium**

Component	Concentration (μM)
NAA (α-naphthaleneacetic acid)	1.07
Kinetin	0.93
BA (6-benzyladenine)	0.88
Ancymidol	3.90

**Table 3**  
**Growth regulators for shoot multiplication medium**

Component	Concentration ( $\mu\text{M}$ )
NAA	1.07
Kinetin	0.93
BA	0.44
Ancymidol	0.39

**Table 4**  
**Growth regulators for root formation medium**

Component	Concentration ( $\mu\text{M}$ )
NAA	1.07
Ancymidol	5.07

### 3. Methods

The prerequisite of plant tissue culture is to establish and maintain fully aseptic conditions. This can be achieved by sterilization of all working tools, media, explants, and care is taken to maintain sterile working environment. Stock solutions of plant growth regulators for culture media should be prepared afresh each time and in small quantities. To avoid precipitation, store all stock solutions at 4 °C.

#### **3.1. Preparation and Sterilization of Culture Media**

1. Use MS basal medium, supplemented with appropriate plant growth regulators (see Subheading 2.3; Tables 1, 2, 3 and 4) to prepare shoot initiation, shoot multiplication, and root formation media. All three media contain ancymidol (storage temperature: 2–60 °C, soluble in DMSO, sterilize with autoclaving, FW: 256.3,  $\alpha$ -cyclopropyl- $\alpha$ -[4-methoxyphenyl]-5-pyrimidinemethanol, moderate acute toxicity; see Note 2).
2. Adjust the pH of the medium to  $5.7 \pm 0.1$  with the addition of 0.1–1.0 M NaOH or HCl.
3. Add agar (7 g/L) and dissolve the mixture in a microwave oven (three times to boiling).
4. Disperse 30 mL medium in baby food jars (6×7 cm) or Magenta® boxes (8×11 cm).
5. Autoclave the medium at 121 °C, pressure 1.2 kg/cm<sup>2</sup> for 20 min.

### **3.2. Plant Source**

#### **Material and Surface Sterilization**

1. Take stem nodes from parental stock plants. It is recommended to grow them in the greenhouse to minimize contamination.
2. Excise stem nodal sections (5–10 cm long) from the upper part of a young spear and wash them for a few minutes under running tap water.
3. Add 0.83 g dichloro-isocyanuric acid (Sigma) to 50 mL reverse osmosis autoclaved water, thoroughly dissolve by mixing and then add a few drops of detergent Tween-20 for better gripping.
4. Surface sterilize spear segments by immersion in 70% ethanol for 1 min, followed by immersion in dichloro-isocyanuric acid solution for 20 min. Mix thoroughly continuously with a magnetic stirrer.
5. Wash explants four times with the sterile reverse-osmosis water and rinse them each time in water for 1–2 min. A sieve can be used for washing, to hold the explants.
6. Excise aseptically the lateral and apical buds (1–3 mm long), including some primordial leaves, from the spear segments and use them as explants (Fig. 1). All procedures should be carried out in laminar hood for aseptic work, using sterilized scalpel and forceps.

#### **3.3. Culture and Maintenance of Explants**

1. Place 5–10 bud explants on the surface of the shoot initiation medium in the baby food jars or Magenta® boxes and seal the jars with parafilm.
2. Grow and maintain cultures in plant growth chamber at  $25 \pm 1$  °C under a photoperiod of 16 h, supplied by Cool White fluorescent light at  $50 \mu\text{mol}/\text{m}^2/\text{s}$ .
3. After the first 4–5 weeks of culture, new shoot structures and crowns are initiated (mean of 4 shoots per explant). At the end of this period, some roots are also formed (Fig. 2; see Note 3).
4. Transfer the shoot cultures on fresh shoot multiplication medium at every 5-week interval to maintain active growth and to promote development and growth of new shoots.
5. Subdivide crowns formed in the first shoot initiation step and use parts containing a crown base and at least two elongated shoots for subculturing on the fresh medium (Fig. 2).
6. In each subculture, discard vitrified and non-vigorous shoots; maintain only healthy shoots for subsequent subcultures.
7. Remove callus formed at the crown base.
8. Cut off apical parts of the shoots to interrupt apical dominance and consequently promote development of the shoots at the base (Fig. 3). During later transfers, subculture explants into the Magenta® boxes containing 30 mL shoot multiplication medium.



Fig. 1. Aseptically excised lateral and apical buds (1–3 mm in length), including some primordial leaves, from the spear segments, used as explants.



Fig. 2. New shoot structures and crowns initiated after the first 4–5 weeks of culture.



Fig. 3. Multiple shoots developed from the shoot tips, which were dissected and subcultured on fresh shoot multiplication medium.

### **3.4. Rooting of *In Vitro* Shoots**

For rooting, divide shoot clusters with well-developed shoots (3–4 cm long) in such a way that they contain crown base (proliferative tissue and callus) with 2–3 shoots. The external callus should be removed, so that newly developed roots gain direct contact with the shoot.

1. Insert the bases of shoots into 30 mL aliquots of MS rooting medium (see Note 4).
2. Incubate cultures at  $25 \pm 1$  °C under a photoperiod of 16 h supplied by Cool White fluorescent light at  $50 \mu\text{mol}/\text{m}^2/\text{s}$ .
3. After 5–6 weeks, the regenerated shoots will develop a root system consisting of some long roots and smaller lateral roots (Fig. 4; see Note 5).

### **3.5. Acclimatization of Plantlets to *Ex Vitro* Conditions**

1. Remove plantlets from the rooting medium and wash them thoroughly under the running tap water in order to remove all agar medium sticking to the roots.
2. Transplant the rooted plantlets to 12 cm pots containing growth substrate (Klasmann Tonsubstrat). Trim shoots to 1 cm to decrease evaporation, immediately after transplanting (see Note 6).
3. Transfer plants to the greenhouse, maintained at  $24 \pm 2$  °C, relative humidity around 80% (see Note 7), with regular watering and nurturing until they are ready for transplanting (Fig. 5).

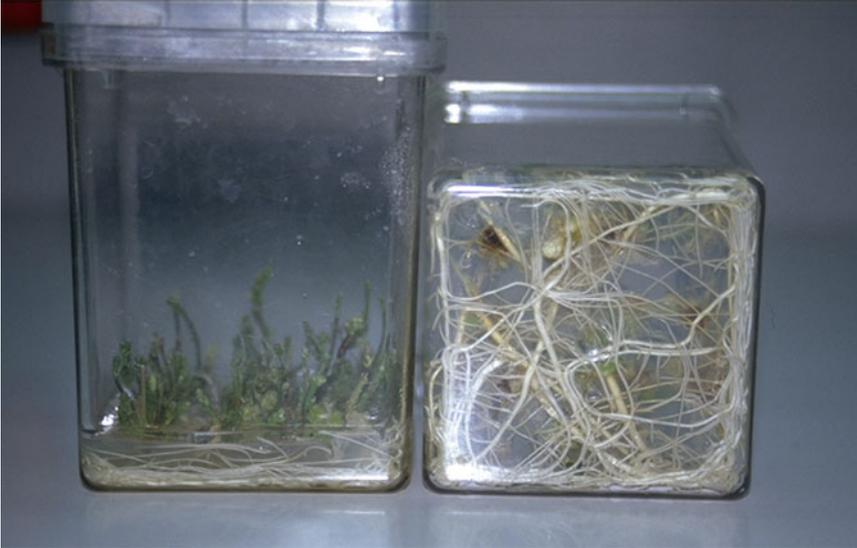


Fig. 4. Developed root system consisting of some long roots and smaller lateral roots.



Fig. 5. Plants after acclimatization in the greenhouse.

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## 4. Notes

1. The micro-incinerator is designed to sterilize metal inoculating loops, needles, forceps, and scalpels without using an open flame; eliminating aerosaling of the microorganisms. By using infrared heat inside a ceramic tube, the micro-incinerator



Fig. 6. Well-developed roots of *Asparagus* plants after acclimatization.

protects from dangerous gases, flames, and splatter. After reaching to optimum sterilization temperature 815 °C (1,500 °F), the instruments are sterilized within 5–7 s.

2. It has been suggested that ancymidol greatly reduces time for the production of transplantable plantlets from about 20 to 8 weeks; and regulate shoot growth, root formation of *Asparagus*. It also promotes stronger shoot and root development and suppresses callus formation (14). Ancymidol is an inhibitor of gibberellin ( $GA_3$ ) synthesis and block the oxidation of  $GA_3$  precursors (13). *Asparagus*-cultured tissues may contain high endogenous  $GA_3$  that inhibits both shoot and root formation.
3. Crown formation, essential for further subculturing, was positively correlated with the number of shoots. Some rooting was also observed on shoot initiation medium; however, those roots were mostly thin and short and such plantlets did not survive acclimatization.
4. The rooting frequency was 82% in the presence of 5.07  $\mu$ M ancymidol.
5. A correlation was made between all measured characters and the most significant positive correlation was obtained between in vitro-grown root length and the number of ex vitro shoots formed per plant. This result demonstrates the importance of root conditions at the acclimatization stage and its dependence on the composition of rooting media (Fig. 6).

6. The efficiency of acclimatization and formation of new ex vitro-grown shoots were examined 5 weeks after transfer of plants from in vitro culture to the greenhouse. The acclimatization of plants was effective (87%), but the number of shoots formed per plant ranged from 2.3 to 12.4. The trimming of shoots at the start of acclimatization benefitted the plant growth, whereas non-trimmed plants ceased growth.
7. The comparison of the photosynthesis and transpiration efficiency of in vitro-grown and acclimatized plantlets showed that two major factors, inefficient stomatal control and excessive water loss, caused death and slow recovery of micropropagated *A. officinalis* during acclimatization (16).

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## Micropropagation and Cryopreservation of Garlic (*Allium sativum* L.)

E.R. Joachim Keller and Angelika Senula

### Abstract

Garlic (*Allium sativum* L.) is a very important medicinal and spice plant. It is conventionally propagated by daughter bulbs (“cloves”) and bulbils from the flower head. Micropropagation is used for speeding up the vegetative propagation mainly using the advantage to produce higher numbers of healthy plants free of viruses, which have higher yield than infected material. Using primary explants from bulbs and/or bulbils (shoot tips) or unripe inflorescence bases, *in vitro* cultures are initiated on MS-based media containing auxins, e.g., naphthalene acetic acid, and cytokinins, e.g., 6- $\gamma$ - $\gamma$ -(dimethylallylaminopurine) (2iP). Rooting is accompanying leaf formation. It does not need special culture phases. The main micropropagation methods rely on growth of already formed meristems. Long-term storage of micropropagated material, cryopreservation, is well-developed to maintain germplasm. The main method is vitrification using the cryoprotectant mixture PVS3.

**Key words:** *Allium sativum*, Cryopreservation, Medicinal plants, Shoot tips, Spices

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### 1. Introduction

Garlic is one of the most ancient cultivated plants of the world. Its uses have been described already in the antiquity (1). It contains healthy compounds, namely sulfur-containing substances, the cysteine sulphoxides, and smells very strong (2). Also together with some other ingredients such as fructans, steroid saponins, and flavonoids, they make garlic in high demand as one of the most important medicinal plants, which is consumed as spice, fresh, and in the processed form. Garlic is worldwide used especially in the Asian cuisine, some European countries, and Latin America. Many health benefits are reported such as antibiotic activities, beneficial

cardiovascular effects, effects on respiratory system, and reducing metabolic diseases, as well as anticancer influences (3).

Almost all garlic genotypes have lost ability to form seeds; a part of the subgroups of this species is forming bulbils in the flower head, whereas the flower buds die off before opening. Another part of the genotypes lost even the ability to form inflorescences (4). Since long time, garlic is being produced by vegetative propagation for the production of clone plants (1). In vitro culture approach, especially micropropagation, has enhanced the efficiency of garlic propagation (5, 6) as well as to produce virus-free plants by meristem culture, thermo-, and/or chemotherapy. Virus-free plants have higher yield (7–10).

Micropropagation (Fig. 1) of garlic has special features because of its bulbous nature (Fig. 2). The meristem region is hidden in the inner part of the bulb at its basal plate. Its morphology and physiology are dependent on the season, late winter, and early spring being the best time to take explants. Additionally, garlic offers some other options for primary explants such as the basal part of young inflorescences, which can be cut into pieces. They regenerate best when some primordia are included on top of the explant (Fig. 3). Bulbils, developing in inflorescences of types which are able to do this, can be used directly as explant sources (11). They grow best at the very young stages during summer and in later stages until spring of next year. Occasionally dormancy occurs, which can be broken by cutting the bulbils. This additional option is delimited to a part of the garlic gene pool, because only the so-called bolting garlic develops inflorescence stalks (4), whereas the other material can be introduced by basal plate and bulb scale explants only. Furthermore, callus cultures or embryogenic cell cultures derived from basal plates, shoot bases, root tips, or leaf bases were used for micropropagation via solid culture, bioreactors, or temporary immersion culture (12–14). Micropropagation is also used for plants obtained from virus elimination by meristem culture (Fig. 4a) (see Note 1).

Another application of micropropagation is the delivery of high numbers of clonal plant material for cryopreservation (Fig. 4b, c). Cryopreservation is the storage of germplasm at very low temperatures in (at  $-196^{\circ}\text{C}$ ) or above (between  $-135$  and  $>-196^{\circ}\text{C}$ ) liquid nitrogen by using cryoprotective substances (Table 1). At such low temperatures, no metabolism and no decay take place and there is no risk of genetic changes. Therefore, the storage may be extended for very long time. Thus, cryopreservation is the safest and the most cost-effective method to store germplasm of only vegetatively propagated crops such as garlic. Maintenance of garlic germplasm via cryopreservation is increasingly applied worldwide (15–19).

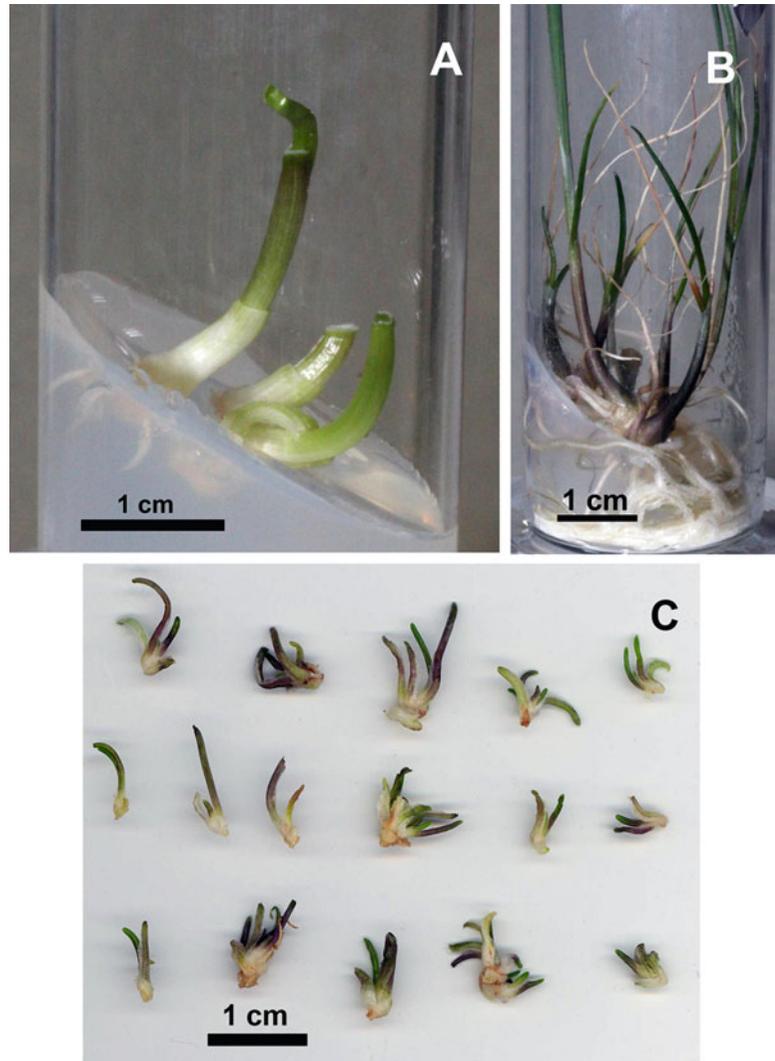


Fig. 1. Main stages of garlic micropropagation from cloves or bulbils: (a) single shoot, already developing new roots, 7 days after transfer to in vitro conditions. (b) Bunch of shoots with well-developed roots, ready to be cut for the next multiplication step. (c) A series of explants derived from a bunch and ready for the next subculture.

## 2. Materials

### 2.1. Surface Sterilization of Source Material and Explant Preparation

1. Tap water.
2. Ethanol 70% (v/v).
3. Sodium hypochlorite solution [NaClO] 12% Cl (Carl Roth GmbH & Co., Karlsruhe, Germany), diluted 1:4 (v:v) with de-ionized water.
4. Surface detergent Tween 20 (Riedel-de-Haën AG, Seelze, Germany).

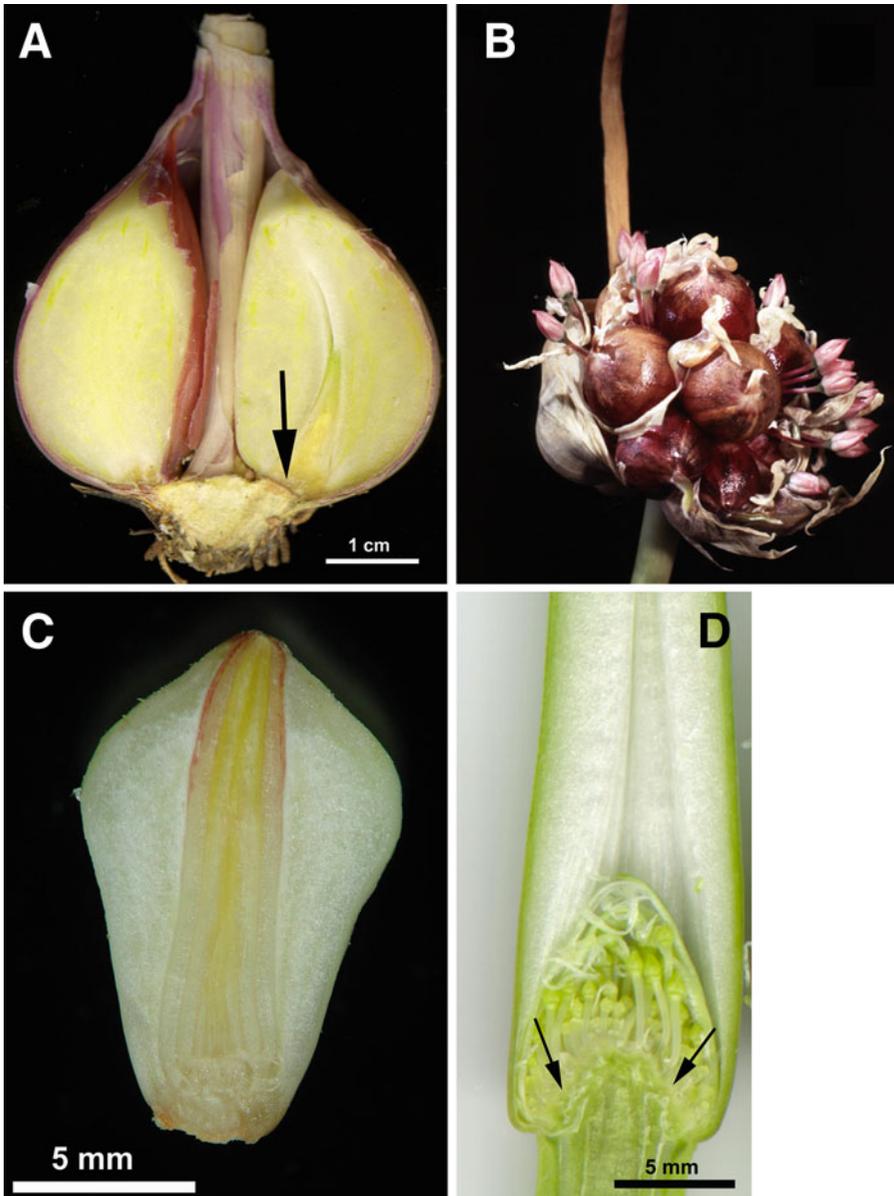


Fig. 2. Main source organs for in vitro culture of garlic. (a) Parts of the compound bulb (“cloves”) are used. The meristem is situated at the basal plate, close to the bottom of the clove (*arrow*). It is covered by several bases of foliage leaf primordia and the thick storage leaf which does not bear a leaf blade. (b) Bulbils in the inflorescence are formed by a part of the garlic genepool only. They vary very much in number and size depending on the genotype. (c) A longitudinal cut of a bulbil shows that it is, basically, structured similarly to cloves. (d) Longitudinal cut of an unripe inflorescence showing spathe, flower primordia, bracts, and bulbil initials. The region marked by *arrows* is still in a meristematic stage and able to regenerate shoots derived from bulbil initials.

5. Autoclaved deionized water.
6. Autoclaved filter paper circles, type 598, diameter 90 mm (Schleicher & Schuell GmbH, Dassel, Germany).
7. Erlenmeyer flasks, 100 mL.

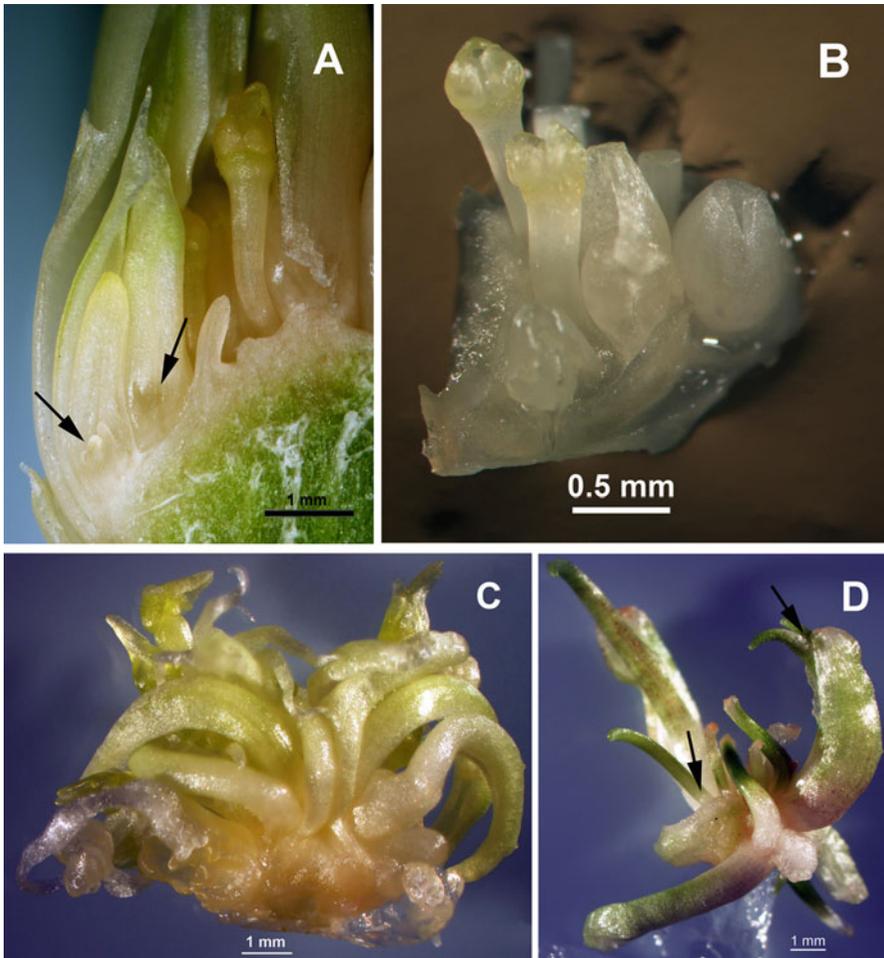


Fig. 3. Main stages of garlic micropropagation from young inflorescence bases: (a) detail of an inflorescence basis, showing the bulbil initials (*arrows*) and a flower primordium. (b) Fresh explant containing bulbil initials and flower primordia. (c) First stage, 14 days after the start of subculture, showing the growth of bracts and leafy structures. (d) Later stage: young shoots appear in the axils of the bracts (*arrows*), developing from bulbil primordia.

8. Tissue culture facilities—Instruments (scalpel, forceps, gas safety burner (schuett phoenix, schuett-biotec GmbH, Göttingen, Germany) to flame culture tubes, glass bead sterilizer (Microsteril 439, C.B.M. S.r.l., Torre Picenardi, Italy) to sterilize instruments), laminar flow bench, culture room.
9. Dissection binocular.
10. Bulbs, cut unripe inflorescences, or bulbils from inflorescences to be used as sources for explants.

## 2.2. Subculturing

1. Autoclaved filter paper discs, grade 3 hw, diameter 125 mm (Munktell & Filtrak GmbH, Bärenstein, Germany).
2. Tissue culture facilities—Instruments (scalpel, forceps, gas safety burner (schuett phoenix, schuett-biotec GmbH,

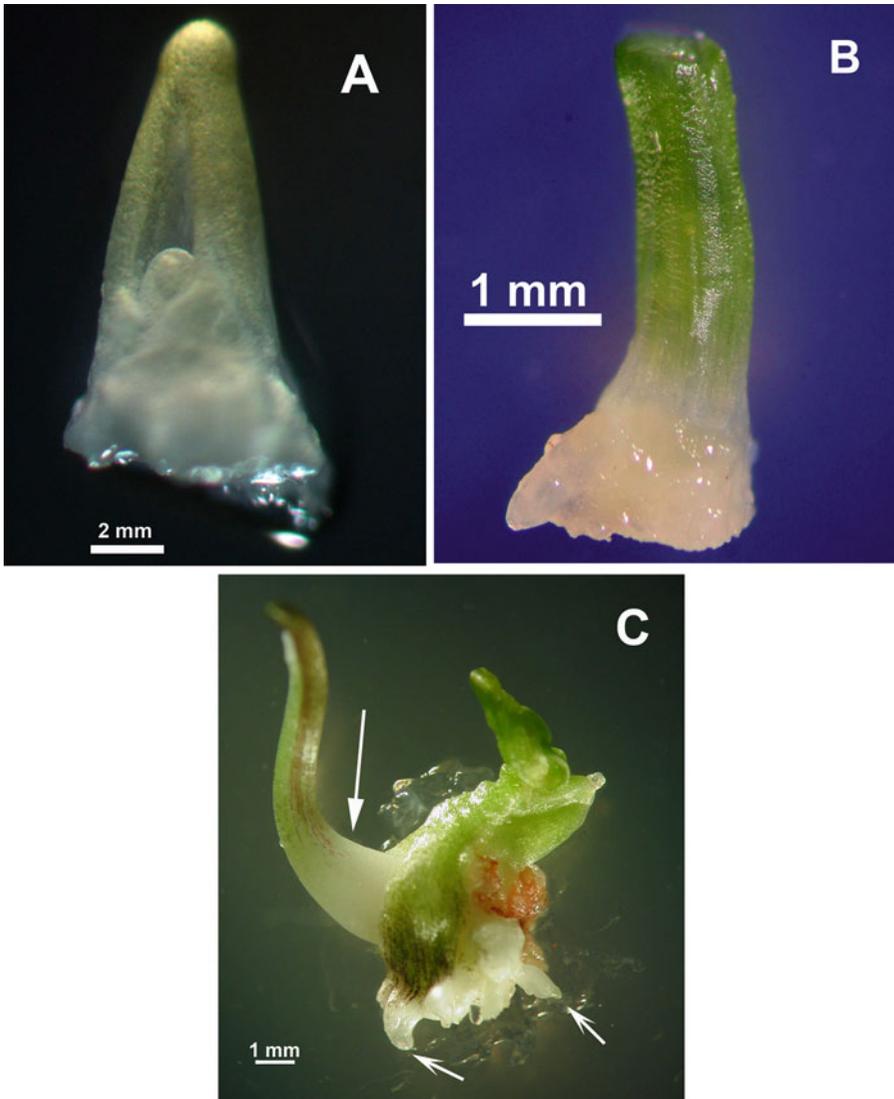


Fig. 4. Examples of explants used for specific applications. (a) Explant used for meristem culture to eliminate viruses; (b) Primary explant used for cryopreservation. (c) First developmental stage after rewarming from cryopreservation, which shows clear evidence of successful regeneration: a developing shoot (*flat arrow*) and root initials (*acute-angle arrows*).

Göttingen, Germany) to flame culture tubes, glass bead sterilizer (Microsteril 439, C.B.M. S.r.l., Torre Picenardi, Italy) to sterilize instruments), laminar flow bench, culture room.

3. Glass Petri dishes (15 cm diameter).
4. Culture tubes 3 cm diameter, 10 cm long.
5. Aluminum foil caps, thickness 50  $\mu\text{m}$ .
6. In vitro cultures of the former subculture as sources of explants.

**Table 1**  
**Survey on cryoprotectants and their mixtures (PVS) usable for cryopreservation of garlic**

Solution	Sucrose (%)	Glycerol (%)	Ethylene glycol (%)	DMSO (%)	Literature
Loading solution A	13.7	18.4	–	–	(21)
PVS2	13.7	30	15	15	(29)
PVS3	50	50	–	–	(22)
PVS4	20	35	20	–	(30)
No name	–	–	–	10	(16, 31)

### **2.3. Culture Media and Their Constituents**

1. Media based on the formulation of Murashige and Skoog—MS (20), standardized powder containing macro- and micro-elements including vitamins (Duchefa Biochemie B.V., Haarlem, Netherlands).
2. Sucrose (Duchefa Biochemie B.V., Haarlem, Netherlands).
3. Agar Agar Kobe I (Serva Electrophoresis GmbH, Heidelberg, Germany).
4. 1-Naphthalene acetic acid (NAA; Duchefa Biochemie B.V., Haarlem, Netherlands).
5. 6- $\gamma$ - $\gamma$ -(dimethylallyl)aminopurine (2iP; Duchefa Biochemie B.V., Haarlem, Netherlands).
6. Standard medium consisting of MS+0.1 mg/L NAA+0.5 mg/L 2iP, 3% sucrose, 1% agar, pH 5.8 (see Note 2).
7. Bacteria screening medium 523 (Duchefa Biochemie B.V., Haarlem, Netherlands) (see Note 3).

### **2.4. Acclimatization of Regenerated Plants to Ex Vitro Conditions**

1. Plastic pots (9 cm diameter).
2. Potting medium consisting of sand, Klasmann substrate 1, steamed soil mixture in proportion 1:2:1 by volume.
  - Klasmann substrate 1 from Klasmann-Deilmann GmbH, Geeste, Germany.
  - Steamed soil mixture consisting of compost, sand (0–2 mm grain size), and white peat in proportion 1:1:1.
3. Previcur® Bayer CropScience Langenfeld, Germany.

**2.5. Special  
Equipment, Material,  
and Media for  
Cryopreservation**

1. Cryotank (e.g., MVE CryoSystem 6000, for 6,000 cryotubes; Jutta Ohst german-cryo<sup>®</sup> GmbH, Jüchen, Germany).
2. Cryotubes 1.8 mL NUNC CryoTubes<sup>™</sup> with internal thread and starfoot (Nunc GmbH & Co. KG, Langenselbold, Germany).
3. Boxes for cryotubes (Nunc GmbH & Co. KG, Langenselbold, Germany).
4. Special labels, resistant to liquid nitrogen.
5. Label printer.
6. Dewar vessel for liquid nitrogen.
7. Water bath (IKA<sup>®</sup> Werke GmbH & Co. KG, Staufen, Germany).
8. Liquid nitrogen.
9. Petri dishes (6 cm diameter) (Nunc GmbH & Co. KG, Langenselbold, Germany).
10. Parafilm (Duchefa Biochemie B.V., Haarlem, Netherlands).
11. Preculture medium: 10% (v/w) sucrose in medium MS (21).
12. Loading solution: 13.7% (v/w) sucrose + 18.4% (v/w) glycerol (21).
13. Plant Vitrification Solution (PVS3) 50% (v/w) sucrose + 50% (v/w) glycerol (22).
14. Washing solution: 41% (v/w) sucrose in medium MS.
15. Carbenicillin (Duchefa Biochemie B.V., Haarlem, Netherlands) (see Note 3).
16. Micropropagated in vitro plants.

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### 3. Methods

Micropropagation of garlic relies on shoot regeneration, which can be obtained from three different sources: (i) bulb basal plates, (ii) young inflorescence bases, and (iii) bulbils. On the basal plate of the main bulbs, some millimeters apart from their bottom, meristems are situated which can be excised and used. The other two options are restricted to genotypes which did not lose the ability to form inflorescences in the course of domestication, the so-called bolting garlic. The basal part of young inflorescences bearing floral and bulbil primordia is cut into small pieces. Later during the year, bulbils are formed between the pedicels of the later dying flower buds. The bulbils can be used directly after harvest or after storage. Development of first shoots needs some time (1–2 weeks). Two to six weeks later, bunches of shoots are formed, which need to be carefully separated. Whenever spontaneous bunches are not sufficient,

they can be induced by longitudinal cutting. There is no special rooting phase needed, because the roots develop together with the new leaf primordia.

### **3.1. Preparation and Sterilization of Culture Media**

1. Prepare Murashige and Skoog (20) culture media for shoot regeneration from shoot tips, meristems, basal plates, and inflorescences. The shoot regeneration medium contains 0.1 mg/L NAA and 0.5 mg/L 2iP.
2. For convenience, use powdered medium sourced from a commercial supplier (e.g., Duchefa Biochemie B.V., Haarlem, Netherlands). Check the formulation of the commercial preparation.
3. Add other components, such as sucrose and growth regulators, as required. Add the appropriate volume of growth regulators from 0.1 mg/mL stock solution to give the required final concentrations.
4. Adjust the pH of the media to 5.8 using 1 M HCl or 1 M KOH.
5. Add 1% (w:v) Agar Agar Kobe I (Serva Electrophoresis GmbH, Heidelberg, Germany).
6. Dispense the media into suitable containers, e.g., 10 mL aliquots into 30 × 100 mm tubes, or 70 mL aliquots into conserve jars of Ø7 and 10 cm high. Use tubes for the initial stages of culture and jars for the maintenance of shoots.
7. Sterilize the media by autoclaving at 121°C for 20 min (118 kPa steam pressure).
8. Store the autoclaved media at 10°C for a maximum of 2 months. Before using the media must be warmed up to room temperature.

### **3.2. Plant Source Material and Surface Sterilization**

#### *3.2.1. Source 1: Basal Plate*

1. Harvest garlic bulbs, when ripe, (June–July in the Northern Hemisphere).
2. Dry the bulbs on shelves in good ventilation to avoid moulding and clean them from soil when dry.
3. Store the bulbs until use under conditions of good ventilation at 4–10°C (see Note 4).
4. Before use separate the cloves and remove the outer scale.
5. Remove the outer part of the thick storage scale and trim the basal inner part to a cube of 10 mm size containing the basal plate and the meristem.
6. Rinse this part under running tap water for few seconds.
7. Prepare the sodium hypochlorite solution as described in Subheading 2.1 and add a drop of Tween 20.
8. Perform the surface sterilization of this part. Begin with shaking in 70% ethanol for 10–20 s.

9. Then shake the pieces in the hypochlorite solution for 20–30 min.
10. Wash the pieces four to five times with sterile water.
11. Prepare the final explants (2–3 mm thick, 3–5 mm long) by cutting with a sharp scalpel under the dissection microscope.
12. Transfer the explants on the shoot culture medium and cultivate them at 20–25°C and 16 h illumination of 60–80  $\mu\text{mol}/\text{m}^2/\text{s}$ .

### 3.2.2. Source 2: Young Inflorescences

1. Harvest unripe inflorescences and store them for 4–6 weeks in a refrigerator at 4°C.
2. Cut the inflorescences, and sterilize them when the spathe is still closed.
3. Prepare the sodium hypochlorite solution as described in Subheading 2.1 and add a drop of Tween 20.
4. Perform the surface sterilization of the inflorescences. Begin with shaking in 70% ethanol for 10–20 s.
5. Then shake them in the hypochlorite solution for 15 min.
6. Wash the inflorescences four to five times with sterile water.
7. Remove the spathe and trim the inflorescences by removing the flower buds and pedicels and longer leaf primordia so that the inflorescence bases remain.
8. Cut the inflorescence bases longitudinally in parts 5 × 5 mm (halves of quarters depending on the size).
9. Transfer the cuttings on culture medium and culture them at 20–25°C and 16 h illumination of 60–80  $\mu\text{mol}/\text{m}^2/\text{s}$ .

### 3.2.3. Source 3: Bulbils

1. Harvest garlic bulbils when ripe, dry and clean them and store them until use at 4–10°C.
2. Remove the outer scale of the bulbils and sterilize them (see Note 5).
3. Prepare sodium hypochlorite solution as described in Subheading 2.1 and add a drop of Tween 20.
4. Perform surface sterilization of this part. Begin with shaking in 70% ethanol for 10–20 s.
5. Shake bulbils in the hypochlorite solution for 20–30 min.
6. Wash bulbils four to five times with sterile water.
7. Prepare final explants (2–3 mm diameter) by longitudinal cuts by using a dissection microscope (see Note 6).
8. Transfer explants on the culture medium and cultivate them at 20–25°C and 16 h illumination of 60–80  $\mu\text{mol}/\text{m}^2/\text{s}$ .

### **3.3. Culture and Maintenance of Explants**

1. In most cases, the duration of a subculture should be 6–8 weeks. This, however, depends on the genotype, and some types will need longer time.
2. Take bunches of multiple shoots out of the culture vessel; remove all dead parts (leaf sheaths) and roots.
3. Cut bunches longitudinally into smaller parts of 3–5 shoots (see Note 7).
4. Trim the shoots to about 1 cm long and the roots to 2–3 mm.
5. Single shoots which are thick enough (2–3 mm diameter) are cut longitudinally after trimming them to 1 cm long.
6. Place these explants into culture tubes and wait for growth of new shoots.
7. Treat new bunches of shoots as under step 3, thus forming an iterative process.
8. Check the cultures regularly and discard contaminated and hyperhydric plantlets (see Notes 3 and 8).

### **3.4. Acclimatization of Regenerated Shoots to Ex Vitro Conditions**

1. Remove the rooted shoots from the culture medium (see Note 9).
2. Transfer plants to 9 cm diameter plastic pots (5 plants/pot) each containing a mixture of sand, Klasmann substrate 1, and a soil mixture in proportion 1:2:1 by volume.
3. Water the pots thoroughly with 0.15% Previcur solution, prepared in tap water.
4. Cover the plants by a transparent plastic hood on the greenhouse table at 18–20°C.
5. After 7 days, remove the hood and, after another week, transfer the pots into lower temperature (10–16°C).

### **3.5. Cryopreservation of In Vitro Multiplied Garlic**

#### *3.5.1. Preculture of the In Vitro Donor Plants*

Subculture plantlets with well-developed roots (avoid to use bunches or clumps of shoots) on standard medium (see Subheading 2.3) for 6–8 weeks at alternating temperatures (optimum day/night temperature 25/–1°C and 16 h light) (see Notes 10 and 11).

#### *3.5.2. Preparation and Pretreatment*

1. Take explants from single, well-developed in vitro plantlets.
2. Take plantlets out of the vessel and excise explants of about 1–2 mm thick and 3 mm long.
3. Preculture explants on the standard medium containing 10% sucrose in Petri dishes sealed with Parafilm for 20–24 h at 25°C.
4. Loading Phase: transfer explants into cryotubes (10 explants/tube), add 1 mL loading solution (see Subheading 2.5) to the

tubes, close tubes and shake them, place tubes at ambient temperature for 20 min, and remove loading solution.

5. Dehydration: add 1 mL PVS3 (see Subheading 2.5) to the tubes; close the tubes and shake them; place the tubes at ambient temperature for 2 h, remove PVS3 solution.
6. Transfer to liquid nitrogen: add 0.5 mL PVS3 solution per tube, close tubes, and shake them, then plunge them immediately into a Dewar vessel with liquid nitrogen.
7. Transfer the cryotubes to be stored into the liquid nitrogen tank.

### 3.5.3. Rewarming and Regeneration

1. Rewarm tubes quickly in a water bath at 40°C for 2–2.5 min (see Note 12).
2. Remove PVS3 solution.
3. Washing: add 1 mL washing solution (see Subheading 2.5), close the tubes and shake them, place the tubes at ambient temperature for 10 min.
4. Remove liquid medium, place the explants shortly on a filter paper, transfer explants into Petri dishes with solid standard medium 3% sucrose + 500 mg/L Carbenicillin, and seal the Petri dishes with Parafilm.
5. Allow explants to grow on the standard medium in the Petri dishes, in the dark at 25°C for 7 days.
6. Thereafter, further cultivate the Petri dishes in 16 h light at 20–25°C.
7. Fourteen days after rewarming, transfer explants to tubes containing standard medium without Carbenicillin.

### 3.5.4. Counting the Results (Growth Controls)

1. Take first observation 14 days after rewarming, count the survival of explants.
2. Transfer the explants into culture vessels containing standard medium.
3. Take second observation 7–10 weeks after rewarming, count the regeneration of small plantlets (see Note 13).

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## 4. Notes

1. Field collections of vegetatively propagated plants are usually infected with virus diseases, and garlic is one of them. Under the European conditions, four main viruses are most abundant (OYDV—onion yellow dwarf virus; LYSV—leek yellow stripe virus; GCLV—garlic common latent virus; SLV—shallot latent virus)

as well as various species of allexi viruses. To remove viruses from the plants, meristem culture may be applied. It consists of isolation of smaller explants, 0.5 mm in diameter, which contain the meristematic dome and one or two leaf primordia. They are cultivated on medium MS+0.1 mg/L kinetin+0.1 mg/L indole acetic acid (IAA) (5) and are treated later like normal micropropagated plantlets. Virus elimination may be supported by chemotherapy: ribavirin (50 mg/L, autoclaved) is added to the induction medium. Thermotherapy was useful as pretreatment to bulbils (40 days at 36°C), followed by meristem isolation. The presence of viruses needs to be checked at least twice when plants are still in vitro and after transfer into soil, because the elimination rate is not 100%. Viruses can be indexed by ELISA technique or PCR-based molecular analyses. Reliability of virus elimination by cryotherapy has not yet been demonstrated for garlic.

2. Also other hormone combinations were published and proved to be suitable under our conditions
  - 0.1 mg/L kinetin+0.1 mg/L IAA (6).
  - 2 mg/L benzyl adenine (BA)+0.2 mg/L NAA (23).
3. Like most other plants, garlic is colonized by endophytes, mainly bacteria, which cannot be removed by surface sterilization. These hidden organisms are not always visible and may appear in the form of sudden outbreaks of white or yellowish slimy exudates or as a more or less permanent tiny veil surrounding the basis of the explant. It may be tolerated by the plants or, when stronger, may contribute to its death. Culture vessels with strong outbreaks need to be removed. More dangerous is the permanent accumulation of endophytes from subculture to subculture, which is the reason for limitation of micropropagation cycles to duration of several years only and an obstacle for long-term slow growth culture. Use of antibiotics such as carbenicillin (500 mg/L) does not generally remove bacteria, but may help the in vitro plants surviving critical periods (treatments like strong trimming for cryopreservation). Bacteria test media should be always present in case of the need to get information about the general situation of the explants (e.g., for using them in cryopreservation).
4. When no insects or moulds remain inside the compound bulb, the storage is better and bulbils remain intact. Separate cloves immediately before use.
5. Mites are vectors for virus diseases in garlic like in other plants, and directly transport fungal spores leading to the spread of infections. Furthermore, large mite populations weaken the bulbils, so that they become no longer usable. Therefore, bulbils should be healthy as sources for in vitro culture. Make sure

that wheat curl mites do not colonize bulbils below the outer scales.

6. Some genotypes possess very small bulbils. They can be transferred on the culture medium after either transversal cut or without cut.
7. Isolation of single shoots may result in growth retardation or even death of the plantlets. Therefore, small bunches should remain which normally perform better *in vitro*.
8. Hyperhydration is a degenerative phenomenon consisting of aberrant filling of the intercellular spaces of the tissue with water giving the plant a glassy appearance (24–26). It increases with the age of cold-stored cultures. The increase in the ratio of potassium nitrate/ammonium chloride reduces hyperhydration in garlic (27) and cooling the bottom of the culture vessels/shelves is also recommended. The subcultures of cold-stored cultures should not exceed 1 year.
9. Since garlic, like other bulbous monocots, does not easily regenerate roots, do not wash them as harshly as it is recommended for many dicots, when you remove the culture medium. Rather treat roots smoothly and prevent possible fungal development on the remaining medium by a fungicide such as Previcur.
10. If no special incubator is available, culture at constant temperature will also give plant material which regenerates after cryopreservation; however, most often the regeneration rates will be lower.
11. *In vitro* preculture is not necessary in case of direct use of bulbils or cloves. In this case, the procedure starts with the preparation step.
12. Rewarming of a control is always necessary to be sure that the cryopreservation procedure was performed correctly. The regeneration rates give a characteristic figure about genotype dependence and eventual detrimental factors such as endophytes. Statistical considerations were published about the number of explants necessary for secure recovery of at least one living plant from the sample and about the optimum numeric relation between the main sample and the regeneration control (28). Concluding from the calculations given by these authors, the following recommendation is given: Per accession 100 explants must be introduced into storage. For the growth and regeneration control, additionally 50 explants need to be cooled and rewarmed after about 1 h. When regeneration rates are better than 30% (calculated in relation to the number of explants used for the control), the sample is complete. When regeneration rates are between 30 and 10%, a second set of 100 explants needs to be introduced with again 50

explants as control. When the regeneration rates in the sum of both sets are less than 10%, do not use this accession for cryopreservation with the given protocol. For safety reasons, split the sample into two equal parts and place them into two different tanks at least or send them to another cryopreserving institution.

13. Survival is characterized by yellowish-green color, sometimes swelling and limited growth of the explants. Regeneration is characterized by the development of regularly formed shoots and later on roots.

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## Micropropagation of Globe Artichoke (*Cynara cardunculus* L. var. *scolymus*)

Giovanni Iapichino

### Abstract

The globe artichoke (*Cynara cardunculus* L. var. *scolymus*) is a perennial plant cultivated in the Mediterranean region and the Americas for its edible young flower heads. Although vegetative propagation by offshoots or by “ovoli” (underground dormant axillary buds) has been the primary method of propagation, the potential for the diffusion of diseases and the phenotypic variability can be very high. The propagation of this species by axillary shoot proliferation from in vitro-cultured meristems produces systemic pathogen-free plants and a higher multiplication rate as compared to that obtained by conventional agamic multiplication. Axillary shoot proliferation can be induced from excised shoot apices cultured on Murashige and Skoog agar solidified medium supplemented with various concentrations of cytokinins and auxins, depending on genotype. For the production of virus-free plants, meristems, 0.3–0.8 mm long are excised from shoot apices and surface sterilized. The transfer of artichoke microshoots to a medium lacking cytokinins or with low cytokinin concentration is critical for rooting. Adventitious roots develop within 3–5 weeks after transfer to root induction MS medium containing NAA or IAA at various concentrations. However, in vitro rooting frequency rate is dependent on the genotype and the protocol used. Acclimatization of in vitro microshoots having 3–4 roots is successfully accomplished; plantlets develop new roots in ex vitro conditions and continue to grow.

**Key words:** Axillary shoot proliferation, Globe artichoke, Offshoots, Underground buds, Vegetable crops

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### 1. Introduction

Artichoke (*Cynara cardunculus* L. var. *scolymus*) (family Asteraceae), also known as globe artichoke, is a perennial plant, native to the Mediterranean basin. Artichoke edible young flower head or capitulum is consumed worldwide as a fresh, frozen, or canned delicacy. In 2008, the total estimated harvested area of commercially grown artichoke was approximately 126,000 ha worldwide with a total production of 1,386,848 tons (1). The leading producing countries Italy, Spain, and France grow artichoke

in area of 49,952 ha, 16,800 ha, and 9,475 ha, respectively. Other artichoke producing countries are Peru (7,790 ha), Chile (4,996 ha), Argentina (4,700 ha), Egypt (3,800 ha), and the USA (3,560 ha). Artichoke is a cool season field-grown crop. Plants that are very sensitive to frost and buds are damaged at  $-2^{\circ}\text{C}$ . Temperatures below  $-10^{\circ}\text{C}$  for several days are lethal for the fleshy underground stem. Buds are adversely affected at higher temperature, over  $24^{\circ}\text{C}$  (2).

In the beginning of artichoke cultivation, both sexual and vegetative propagation were used. Since the seventeenth century vegetative propagation has been the primary means of propagation, resulting in clones with different adaptation capacity and often characterized by heterogeneity. Nowadays, seed artichoke cultivars, released from commercial seed companies are grown in the USA, Israel, and France (2). Asexual propagation is carried out by crown division, by offshoots, or by “ovoli.” Vegetative propagation material is generally taken from commercial fields at the end of the production cycle. In the first method (crown division), plants are collected and cut in to sections, and each section includes a stem piece and some roots of the original plant. In the second method, rooted offshoots are separated when they emerge from the base of the original plants after the winter hibernation. Finally, “ovoli” are dormant or semidormant underground axillary buds produced on short, swollen woody protuberances. They are separated from the dried stump in early summer and planted in July–August (3).

As artichokes are infected by various diseases such as nematodes, bacteria, fungi, and viruses (4), the reliance on vegetative propagation results in multiplication and spread of infected plants in the fields meant for commercial production. Therefore, plantation of pathogen-free propagation material is a prerequisite to maintain adequate high yields. Tissue culture methods applied for solving artichoke sanitary problems started in Spain in 1974 (5) and continued in Tunisia, where the establishment of a rapid and efficient method of mass propagation and virus eradication through meristem culture was reported (6).

Micropropagation of the globe artichoke has been mainly focused on in vitro shoot culture of European cultivars and to the Green Globe cultivar (7–14). Other attempts using non-meristematic tissues, such as the receptacle, as a starting explant were unsuccessful due to high contamination rate and browning of the tissue caused by phenols (4). Artichoke in vitro rooting and acclimatization are crucial steps. Various protocols to overcome this problem have been developed (15). The primary objective of this manuscript is to illustrate the sequential steps required for the micropropagation of artichoke by shoot culture.

## 2. Materials

### 2.1. Surface Sterilization of Source Material

1. Tap water.
2. Ethanol 70 % (v:v).
3. Commercial bleach solution (1.05 % NaOCl (w/v)).
4. Tween 20 (Sigma Chemical Co., St. Louis, MO, USA).
5. Reverse osmosis water.
6. Magnetic stirrer, magnetic bars.
7. Tissue culture facilities—Instruments (scalpel, forceps, spirit burner to flame, sterilize instruments), laminar flow cabinet, culture room.
8. Selected plant as a source of explants.
9. Citric acid and ascorbic acid solution.

### 2.2. Culture Media

1. Media based on the formulation of Murashige and Skoog (MS) (16) for: (a) meristem culture from shoot apices, (b) in vitro shoot establishment and multiplication by axillary shoot proliferation, and (c) in vitro rooting of microshoots are given in Tables 1, 2, and 3.

**Table 1**  
**Shoot meristem media**

	Harbaoui and Debergh (6)	Ancora et al. (8)	Moncousin (9)	Pecaut et al. (10)	Bigot and Foury (11)	Babes et al. (21)
Macroelements	MS	MS	MS	MS	MS mod	MS
Microelements	Nitsch and Nitsch	MS	Gamborg	MS	MS	MS
Fe EDTA	MS	MS		MS	MS	MS
Vitamin and aminoacids	–	MS		MS	MS	MS
Myo-inositol (mg/L)	100	100				
Thiamine (mg/L)	0.4	–				
Growth regulators (mg/L)						
2-iP	1.0		5.0			
Kin		5.0		1.0	1.0	0.1
BAP						
GA <sub>3</sub>	0.025		0.1			0.05
IAA	1.0	0.5	0.1			
NAA	–			0.1	0.1	
Sucrose (g/L)	20	40	20	30	30	30
Other components (mg/L)						
Adenine			100	40	40	
NaH <sub>2</sub> PO <sub>4</sub>				85		
Agar (%)	0.6	0.7	0.7	NS	0.7	0.8
pH	5.6	5.5	5.7	NS	5.5	5.5

NS Not specified

**Table 2**  
**Shoot proliferation media**

	Harbaoui and Debergh (6)	Ancora et al. (8)	Moncoussin (9)	Pecat et al. (10)	Bigot and Foury (11)	Rossi and De Paoli (12)	Iapichino (19)	Brutti et al. (7)	Tavazza et al. (20)
Macroelements	MS	MS	MS	MS	MS mod	MS mod	MS	MS mod	MS mod
Microelements	Nitsch and Nitsch	MS	Gamborg	MS	MS	MS	MS	Heller	
Fe EDTA	MS	MS	NS	MS	MS	MS	MS	MS	
Vitamin and aminoacids	MS	MS	NS	MS	MS	MS	MS	Gamborg	
Myo-inositol (mg/L)	100	100					100		
Thiamine (mg/L)	0.4								
Growth regulators (mg/L)									
2-IP			10					10	
Kin	2-5	5.0		1.0	1.0			2.0	2.0
BAP						0.05	1.0		
GA <sub>3</sub>	0.01		0.5						
IAA	1.0	0.5	0.1			0.5			
NAA				0.1	0.1			0.5	
Sucrose (g/L)	20	40	20	30	30	20	30	30	
Other components (mg/L)									
Ascorbic acid		10					10		
Adenine			100	40	40			80	
NaH <sub>2</sub> PO <sub>4</sub>				85					
NH <sub>4</sub> NO <sub>3</sub>						400		825	400
Ca(NO <sub>3</sub> ) <sub>2</sub>									1,000
KNO <sub>3</sub>						400		950	800
Agar (%)	0.6	0.7	0.7	NS	0.7	0.7	0.8	0.8	
pH	5.6	5.5	5.7	NS	5.5	5.8	5.7	NS	

NS Not specified

**Table 3**  
**Rooting media**

	Harbaoui and Debergh (6)	Ancora et al. (8)	Moncousin (9)	Pecat et al. (10)	Bigot and Foury (11)	Rossi and De Paoli (12)	Iapichino (19)	Brutti et al. (7)	Tavazza et al. (20)
Macrolements	MS	MS/2	MS/2	MS/2	MS/2	MS mod	MS/2	MS mod	MS mod
Microlements	Nitsch and Nitsch	MS	MS	NS	MS	MS	MS	Heller	
Fe EDTA	MS	MS	NS	NS	MS	MS	MS	MS	
Vitamin and aminoacids	-		NS	NS	MS	MS	MS	Gamborg	
Myo-inositol (mg/L)	100	100	NS	NS			100		
Thiamine (mg/L)	0.4								
Growth regulators (mg/L)									
Kin				0.05					
IBA	2.0								
IAA						2.0	2.0		10
NAA		2.0	0.1	0.5	0.1	2.0		3.0	
Sucrose (g/L)	20	20	20	NS	20	20	30	20	
Other components (mg/L)									
Activated charcoal					2.0				
Cyclodextrins								2,000	
Ascorbic acid		10					10		
L-Tyrosine			50	50					
NaH <sub>2</sub> PO <sub>4</sub>				85					
NH <sub>4</sub> NO <sub>3</sub>						400			400
Ca(NO <sub>3</sub> ) <sub>2</sub>						400			1,000
KNO <sub>3</sub>									800
Agar (%)	0.6	0.7	0.8	NS	0.7	0.7	0.8	0.6	
pH	5.6	5.5	NS	NS	5.5	5.8	5.7	NS	

NS Not specified

2. Powdered medium (Sigma-Aldrich Chemical Co., St. Louis, MO, USA). Check the formulation of the commercial preparation.
3. Test tubes (25×150 mm) (Sigma-Aldrich Chemical Co., St. Louis, MO, USA).
4. GA 7 vessels (77 mm×77 mm×97 mm) (Magenta Corp., Chicago, IL, USA).
5. Agar (Sigma-Aldrich Chemical Co., St. Louis, MO, USA, no. A-1296).

### **2.3. Acclimatization of Regenerated Plants to Ex Vitro Conditions**

1. Tap water.
2. Plastic pots (8 cm diameter).
3. Potting medium consisting of a peat-perlite mixture 4:1 (v/v).
4. Intermittent mist or clear plastic film.

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## **3. Methods**

The protocol described here includes four stages which are: Stage (1) selection of source plant material, meristems or apices of larger size, and establishment of aseptic culture; Stage (2) induction and maintenance of axillary shoot proliferation by transferring Stage 1 microshoots into a medium supplemented with appropriate cytokinin and auxin concentrations; Stage (3) in vitro rooting of microshoots; and Stage (4) plantlet acclimatization.

### **3.1. Preparation and Sterilization of Culture Media**

1. Prepare culture media for (a) meristem culture, (b) in vitro establishment and proliferation of apices of larger size, and (c) in vitro rooting of microshoots according to the formulations given in Tables 1, 2, and 3.

Media containing MS macrosalts are commonly used. The micronutrients of Nitsch and Nitsch (17) and Gamborg et al. (18) media have also been used. The addition of  $\text{NaH}_2\text{PO}_4$  and adenine sulfate in the culture media also benefits the growth of artichoke cultures.

2. Prepare culture media by using commercially available powder culture medium.
3. Add other components, such as sucrose and plant growth regulators (PGR), as required. Add appropriate amount of PGR by using 1.0 mg/mL stock solutions (see Note 1) to raise the final volume of the culture medium by adding distilled water. Media containing low concentrations of PGR are well suited for meristem culture. By culturing apical meristems, shoots regeneration is successful on MS or MS-modified medium

containing 1–5 mg/L 6- $\gamma,\gamma$ -dimethylallylamino purine (2-iP), 1–5 mg/L Kinetin (Kin) and 0.1–1.0 mg/L indole-3-acetic acid (IAA), or 0.1 mg/L  $\alpha$ -naphthaleneacetic acid (NAA). The addition of 0.025–0.1 mg/L gibberellic acid (GA<sub>3</sub>) stimulates elongation and growth of apical meristems into shoots.

High concentrations of PGR are needed when apices of larger sizes are established and multiplied in culture. Selection of PGR concentrations for shoot establishment media is based on explant survival and development and for shoot proliferation media is based on shoot multiplication rate and shoot length. Cytokinins are generally added to the media to induce shoot proliferation. However, depending on the cultivar and clone, addition of auxins may or may not hasten cytokinin-induced axillary shoot multiplication. Shoots were successfully multiplied from shoot apices by culturing on MS or MS-modified medium containing 10 mg/L 2-iP, 1.0–5.0 mg/L Kin, 0.05–1.0 mg/L 6-benzylaminopurine (BAP) and 0.1–1.0 mg/L IAA, or 0.1–0.5 mg/L NAA. Depending on the genotype, NAA, IAA and to a less extent indole-3-butyric acid (IBA) have been used to induce rooting in regenerated microshoots of artichoke. Shoots have been rooted in MS, MS half-strength or MS-modified medium added with 2.0 mg/L IBA, 2.0–10 mg/L IAA or 0.1–3.0 mg/L NAA (4, 8, 10, 12, 14, 19, 20).

4. Adjust the pH of the media to 5.8 with 1 M HCl or 1 M KOH.
5. Add 0.8 % (w:v) agar.  
Dispense media into suitable containers, e.g., 10 mL aliquots into 25 × 150 mm test tubes for the initial stages of meristem and in vitro shoot establishment; and for shoot multiplication and root induction use large containers, e.g., 50 mL aliquots into GA 7 vessels.
6. Sterilize media by autoclaving at 121°C for 20 min (118 kPa steam pressure).
7. Store the autoclaved media at room temperature in the darkness, maximum for 1 month.

### **3.2. Plant Source Material and Surface Sterilization**

1. Production of virus-free plants: excise meristems (0.3–0.8 mm long) from shoot apices under the binocular microscope (see Note 2).
2. In vitro shoot establishment and multiplication: excise apical shoots either from offshoots or by sprouted “ovoli” (see Note 3). Remove external leaves with scalpel in order to obtain shoot tip explants, 5–10 mm long.
3. Rinse explants under running tap water for 10 min.
4. Surface sterilize explants by immersion in 1.05 % NaOCl solution containing 20 drops of Tween 20/L for 20 min with

gentle agitation. This operation and all subsequent steps of the procedure are carried out in the laminar hood.

5. Wash explants three times with a mixture solution containing 100 mg/L each of citric acid and ascorbic acid for the prevention of browning of tissue (see Note 4).

### **3.3. Initial Establishment of Culture, Axillary Shoot Proliferation, and Maintenance of Explants**

1. Using aseptic techniques, place single explants into 25 × 150 mm test tubes containing shoot meristem culture medium or the shoot establishment/proliferation medium, according to the final objective.
2. Gently insert explants inside the upper surface of the medium, and maintain at 25 ± 2°C under a 16 h photoperiod (50 μmol/m<sup>2</sup>/s, White fluorescent light; 18W/33). Fungal contaminants would appear within a few days of culture, whereas bacterial contaminants are visible after 7–10 days; remove contaminated cultures immediately without any delay.
3. At the initial stage of in vitro artichoke culture, the growth is generally slow. The percentage of shoots with normal growth in the establishment medium depends on genotype, medium, and type and concentration of PGR.
4. After initial growth, about 4 weeks, subculture shoots in the shoot proliferation medium. However, depending on the genotype, shoots may or may not develop new axillary shoots in the establishment medium (Fig. 1). During later transfers,

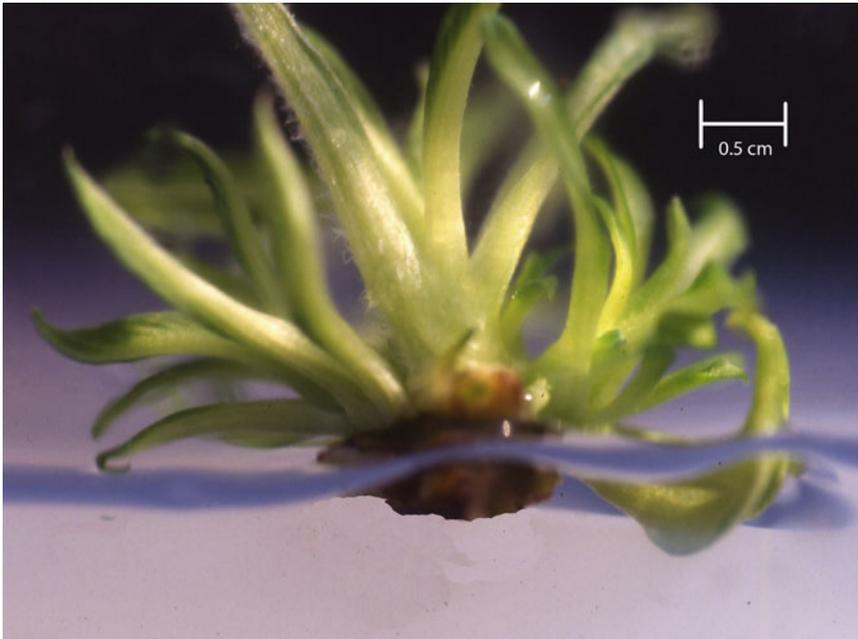


Fig. 1. Micropropagation of *Cynara cardunculus* L. var. *scolymus*. cv. Violetto Spinoso di Sicilia through axillary shoot multiplication on MS medium supplemented with 0.5 mg/L BAP (bar = 0.5 cm).

axillary shoots are subcultured into 50 mL aliquots of shoot proliferation medium in GA 7 vessels.

5. Evaluate shoot multiplication capacity of cultured explants. The average multiplication rate is 2.5–4.0 axillary shoots every 3 weeks for most clones.
6. Transfer single shoots to the fresh medium of the same composition every 4 weeks to maintain active growth.
7. Excise well developed shoots >1 cm long and transfer to MS-based elongation medium without growth regulators or containing 0.05 mg/L 6-benzylaminopurine (BAP) or Kin (see Note 5).

### **3.4. Root Development of In Vitro Regenerated Shoots**

1. Insert the base of microshoots into MS-based root induction medium in 300 mL GA 7 vessels containing 50 mL medium; maintain cultures at  $25 \pm 2^\circ\text{C}$ , 16 h photoperiod ( $50 \mu\text{mol}/\text{m}^2/\text{s}$ , White fluorescent light).
2. After 5 weeks, regenerated roots should be developed at the base of a leaf node. The number of roots vary, 1–4 per microshoot (Fig. 2) (see Note 6). However, the rooting efficiency can further be improved by adding 2 g/L  $\alpha$  or  $\beta$ -cyclodextrins (see Note 7).

### **3.5. Acclimatization of Rooted Microshoots to Ex Vitro Conditions**

1. Remove the rooted microshoots from the rooting induction medium and carefully wash off sticking agar from the roots with warm (approximately  $35^\circ\text{C}$ ) tap water.



Fig. 2. Adventitious root formation on *C. cardunculus* L. var. *scolymus*. cv. Violetto Spinoso di Sicilia microshoot on  $\frac{1}{2}$  MS medium supplemented with 2.0 mg/L indole-3-acetic acid (IAA) (bar = 0.7 cm).

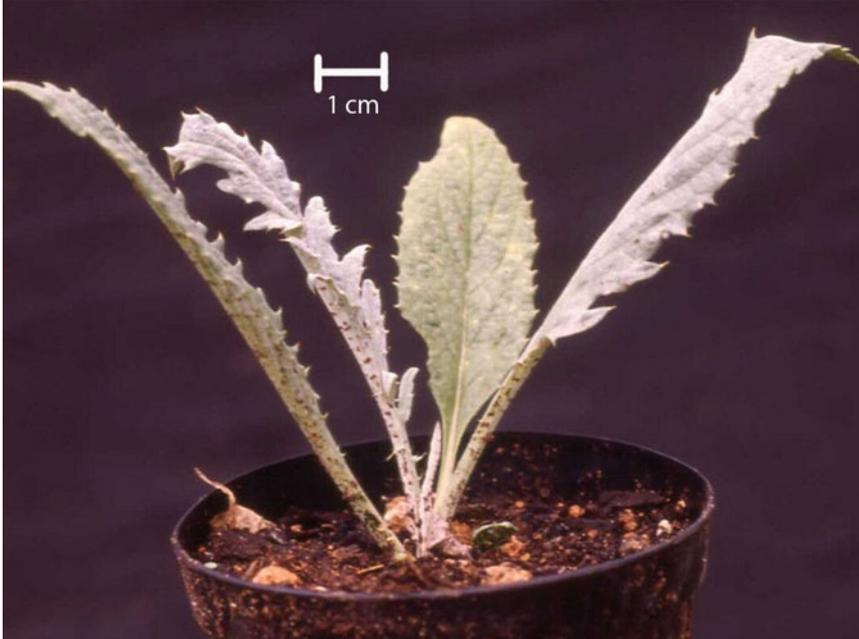


Fig. 3. Plant acclimatized in pot (bar = 1.0 cm).

2. Transfer plants individually to 8 cm diameter plastic pots, each containing a potting medium (see Subheading 2.3, item 3).
3. Maintain the potting medium moist by frequent watering. Keep the plants in the greenhouse covered with 70 % shade cloth at 10–16°C during the night and 18–24°C during the day. Maintain relative humidity close to 80–100 % by placing plants under intermittent mist or clear plastic film.
4. After transfer to ex vitro conditions, rooted plantlets should develop new roots from the root system and continue growth (Fig. 3).
5. After 2 weeks gradually reduce relative humidity.
6. After 4 weeks, transfer plants in a lath house covered with 70 % shade cloth. The lath house night temperature is maintained at around 14–20°C and day temperature 21–27°C, until planted in the field (see Notes 8 and 9).

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#### 4. Notes

1. Prepare separately auxin and cytokinin stock solutions by dissolving 1.0 mg/mL in small volumes (approximately 500  $\mu$ L/100 mg) of 1.0 M KOH or 1.0 M HCl. Adjust the volume with distilled water and store solutions in the darkness at 4°C. Replace stock solutions every 2 weeks.

2. Thermotherapy cycles may be given at different time interval. Infected shoots are multiplied on a basic MS medium containing 0.1 mg/L Kin, 0.05 mg/L gibberellic acid, 30 g/L sucrose, and 8 g/L Bacto-agar. Cultures are pretreated, for about 15 days, by gradually increasing the temperature from 25 to 38°C. Shoot apices 3 cm long are utilized to perform thermotherapy cycles ranging from 22 to 35 days at 38 + 0.5°C with 65 % relative humidity, supplying light 50  $\mu\text{mol}/\text{m}^2/\text{s}$  for 12 h/day. Meristems, 0.3–0.8 mm long, are excised from surviving shoots, transfer on the same medium, and grow for about 30 days. Surviving apices are then transferred and multiplied on the same MS medium supplemented with 0.05 mg/L Kin. This technique may generate up to 69 % virus-free plantlets (12, 21).
3. Explants respond positively to in vitro growth when they are removed in the beginning of their vegetative season: during early summer for the early Mediterranean cultivars and during the spring for the late cultivars.
4. The sterilization of artichoke explants, taken from the field-grown plants, requires great care due to high contamination rate caused by exogenous and endogenous microorganisms. Submerging shoot apices for 30 s in 70 % ethanol prior to soaking in NaOCl solution may improve surface sterilization. Explants turn brown due to leaching of polyphenols in the culture medium. Therefore, citric acid and ascorbic acid are added to the culture media for the prevention/lowering of browning in several artichoke cultivars and clones.
5. The transfer of artichoke microshoots to a culture medium devoid of cytokinins or with low cytokinin concentration before transfer to the rooting medium is critical for rooting (4, 12, 19). The improvement in rooting efficiency can occur onto  $\frac{1}{2}$  strength MS medium without cytokinin (19) or onto an elongation MS-modified medium supplemented with 0.5 mg/L NAA and BA or 0.05 mg/L Kin (12).
6. In vivo rooting of microshoots is also possible (12).
7. The addition of 2.0 g/L  $\alpha$  or  $\beta$ -cyclodextrins to the rooting induction medium supplemented with 3 mg/L NAA can triple the rooting percentage and double the number of differentiated roots per microshoot when compared to the control (7).
8. In vitro rooted plants, 3–4 cm high and with 3–4 roots have high survival rate (12).
9. The agronomic performance (earliness, number of flower heads per plant and weight of flower heads) of in vitro-produced artichoke plants is highly satisfactory due to true-to-type plants in late cultivars used for spring production, such as Romanesco (22), Blanc hyérois, and Camus de Bretagne (23).

However, early Mediterranean cultivars tend to produce untrue types, mainly leading to late bolting plants with more deeply divided leaves. These variants in several early artichoke cultivars have limited the application of micropropagation techniques for spring production (12, 24, 25).

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# **Part IV**

## **Applications of In Vitro Propagation**

## In Vitro Rejuvenation of Woody Species

Paul E. Read and Christina M. Bavougian

### Abstract

Juvenility and phase change in woody plant species exert profound impacts on plant morphology and the ability of explants to be successfully propagated *in vitro*. Morphological characteristics such as leaf shape modifications, thorniness, and the inability to initiate flowers are associated with juvenility. Physiological maturity, that is the ability to reproduce sexually, is reached by many woody species only after many years of juvenile growth. As a result, micropropagation of woody species has historically been difficult with many plant species proving to be exceedingly recalcitrant. The importance of juvenility and its impact on successful vegetative reproduction *in vitro* has therefore received much research attention. In vitro technologies that have been demonstrated to induce rejuvenation include meristem culture, chemical treatments, pruning and hedging, forcing new growth, and taking advantage of epicormic buds, grafting and micrografting, and somatic embryogenesis. Applications of these technologies are discussed in this chapter.

**Key words:** Juvenility, Maturity, Phase change, Woody plants, In vitro culture, Micropropagation, Meristem culture, Somatic embryogenesis

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### 1. Introduction

Multiplication of plants by macropropagation methods such as cuttings is demonstrably easier with juvenile plants than with cuttings taken from mature plants (1). In this chapter, we examine the extrapolation of this concept to *in vitro* methods of mass propagation, especially with woody plant species.

#### 1.1. What Is Juvenility?

Juvenility was described as early as 1900 by Goebel as a physiological condition that may be characterized by morphological characteristics such as leaf shape modifications, thorniness, and the inability of the plant to initiate flowers (2). Juvenility is currently defined as a plant in its vegetative state that is not capable of responding to flower induction stimuli that normally would result in flowering in

an adult plant (3, 4). That is, a plant that is not physiologically mature and therefore incapable of flowering and sexual reproduction. For example, apple trees may not flower and bear fruit for several years following planting. Indeed, the juvenile period may be very long, as in the cases of English oak (*Quercus robur*) and silver fir (*Abies alba*) that exhibit a duration of juvenility of 25–30 years, while European beech (*Fagus sylvatica*) has an even longer juvenile period of 30–40 years. Many other woody plants have juvenile periods of 5–10 or more years, e.g., Scots pine (*Pinus sylvestris*), several birch species (*Betula* spp.), and fruit trees such as apple and pear (5).

Hackett and colleagues (1, 6, 7) have used English ivy (*Hedera helix*) as a model to study juvenility and phase change. Morphological differences become readily apparent when this species changes from the juvenile phase to the adult stage: juvenile *H. helix* exhibit a prostrate growth habit, lobed leaves, and an inherent ability to produce adventitious roots from stems, including the internodes. It also does not flower. However, the adult form has entire leaf margins and erect stems; it may flower, but exhibits recalcitrance with regard to its ability to form adventitious roots. Many explanations have been offered for why cuttings from mature tissues are difficult to root; one explanation involves tissue maturation and formation of lignin in the cells (8), since there is a negative correlation between lignin build-up and the ability to form adventitious roots. Juvenile grapevines cannot flower or produce tendrils (a modified inflorescence) and have spiral (2/5) phyllotaxy as compared to distychous phyllotaxy in mature vines. Mature grapevines normally possess tendrils and exhibit the capacity to flower (9). Juvenile members of the *Citrus* genus often exhibit thorniness, which usually disappears with maturation. Excellent reviews of the phenomenon of juvenility have been presented by Schaffalitzky de Muckadell (10) and Sax (11), with a thorough and detailed discussion provided by Hackett's (12) review of the subject.

The biological basis for phase changes and stability of either phase is not well-understood. It has been postulated to be related to changes during cell division, to hormonal influences, or to cellular isolation, i.e., when meristems are removed from surrounding differentiated cells, or more likely this phenomenon is affected by interactions of numerous physiological and anatomical characteristics of juvenile vs. mature plants or plant parts (12).

### **1.2. Why Is Juvenility Important?**

Practical plant propagators have long been familiar with the ease of propagation of cuttings taken from young (now recognized as juvenile) plants. In 1929, Gardner presented data demonstrating that hardwood cuttings taken from 1-year-old seedlings produced adventitious roots better and faster than cuttings from older plants in 17 of 21 woody species investigated (13). Similarly, Stoutemeyer reported in 1937 on extensive research with many different

genotypes of apple that stem cuttings obtained from apple seedlings in their first year of growth rooted readily (14). However, cuttings from older seedlings rooted poorly or not at all. Stoutemeyer (15), O'Rourke (16), Von Aderkas and Bonga (17), and others have repeatedly shown that phase change is of critical importance to the commercial plant propagator, citing many examples of woody species that are more readily propagated by taking cuttings from juvenile plants or tissues when compared with older or more mature plants or plant parts. This principle has been consistently emphasized over the years by teachers of plant propagation (18, 19) and is further emphasized by Francl et al. (20): "Considering the genetic heterozygosity of most of the forest tree species, cloning is the only method that permits immediate and total capturing of genetic gains." Although they refer to "forest tree species," this statement can be correctly extended to apply to other woody species, such as fruit crops and ornamentals.

### **1.3. How Can Plants Be Rejuvenated?**

There have been numerous reports of procedures demonstrated to induce juvenility in mature (non-juvenile) plants that are recalcitrant when conventional propagation methods are employed. Among them are severe pruning (hedging) (21–24); use of stump sprouts, epicormic shoots, and material from juvenile parts of mature plants (25–28); use of shoots from adventitious buds (29); grafting mature scions onto juvenile rootstocks (30); grafting and sequential grafting (30, 31); treatment of mature plants or plant parts with growth regulating chemicals (32–36); leaf removal from mature trees, thus removing a rooting inhibitor (30, 37); and use of various methods of *in vitro* propagation (12, 38).

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## **2. In Vitro Methods for Inducing Rejuvenation**

Numerous methods have been helpful to researchers studying *in vitro* culture of woody species. In the following sections, we briefly expound upon several of these technologies, providing examples that have been published by an eminent list of scientists who work with *in vitro* propagation.

### **2.1. Meristem Culture**

Some of the earliest research with meristem culture of woody plants was reported by Gautheret (39) in which he cultured *Salix* cambium tissue *in vitro*. Subsequently, Morel and Martin published their classical work with *Dahlia* apical meristems where they were able to eliminate virus through the use of meristem culture, taking advantage of the fact that the virus particles had not yet translocated to the tiny shoot tip (40). Following these reports, ten Houten et al. (41) further elucidated the concept by combining

heat treatment with meristem culture for the production of virus-free plant material. Furthermore, in 1971, Boxus demonstrated successful meristem culture of *Prunus pandora* (42). A key concept critical to success of micropropagation was shown by Cresswell and Nitsch (43) demonstrating the ease of *in vitro* culture of juvenile woody plants, in contrast to the use of explants from mature tissues cultured *in vitro*. Lyrene (44) and others (45) have demonstrated that *in vitro*-derived shoots of blueberry (*Vaccinium* spp.) produce adventitious roots more readily than shoots from conventionally propagated plants. Perhaps the most dramatic example of rejuvenation via meristem culture was reported in 1991 by Monteuiis in which rejuvenation was achieved from *in vitro* culture of a 100-year-old tree of *Sequoiadendron giganteum* (46).

The use of stump or trunk sprouts provided Ball et al. (47) with juvenile *Sequoia* shoots from which explants were successfully cultured *in vitro*. Boulay (48), also working with *in vitro* culture of *Sequoia*, proposed that explants from mature shoots exhibit a “memory,” as explants display plagiotropic shoot orientation. Subsequent subcultures reduced this “memory” so that after several subcultures the number of orthotropic shoots was increased.

Francllet (24) stated that it was impossible to distinguish *in vitro*-derived plantlets of *Sequoia* from seedlings in a nursery setting. Juvenile characteristics such as absence or reduced number of tendrils in “Cabernet Sauvignon” grapevine (*Vitis vinifera*) cultured *in vitro* were diminished by serially subculturing mature shoot tips (9). Similarly, Hartl-Musinov et al. (49) found that tendril production of “Vugava” grapevines cultured *in vitro* was observed in the first subculture but became less numerous with further subcultures, with the tendril number per node essentially zero by the ninth subculture. Spiral phyllotaxy (a juvenile morphological characteristic) was also observed when adventitious shoots were regenerated from petiole explants of “Vugava” grapevine. Francllet et al. (20) found that repeated subcultures with explants from mature cherry trees also led to formation of shoots exhibiting juvenile characteristics. Figure 1 shows rejuvenated *Corylus* hybrids after sequential culture.

## 2.2. Chemical Treatments

As noted earlier for how plants can be rejuvenated, several reports have shown that application of gibberellic acid ( $GA_3$ ) can cause mature *Hedera* plants to change to the juvenile form, whereas abscisic acid (ABA) and growth retardants can stabilize the mature form (32–36). Applications of benzyladenine (BA) to mother plants increased shoot proliferation *in vitro* (50–52). In addition, BA has been employed to “rejuvenate” several woody species, including *Eucalyptus*, *Pinus*, *Sequoia*, and *Vitis* (9, 20, 53–55).

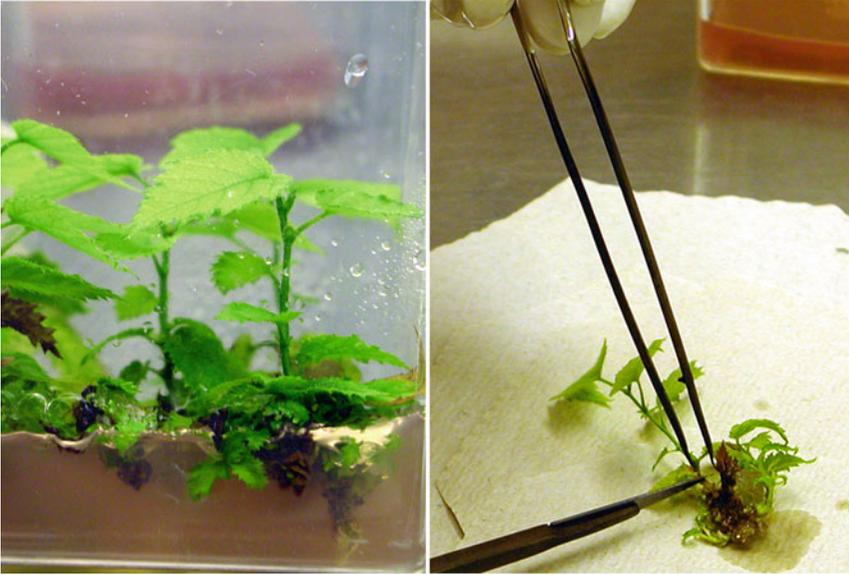


Fig. 1. Following sequential culture of hazelnut (*Corylus* spp.) hybrids, *left*, microcuttings can be readily excised for rooting or for further use as explants to be cultured *in vitro* (*right*).

### 2.3. Pruning/Hedging

As has been the case for propagation by cuttings where pruning mature mother plants gave rise to juvenile shoots that rooted easily, repeated pruning of donor plants has been shown to enhance explant performance *in vitro*. For example, Clapa and Fira (56) used source plant material from *Rhododendron* stock plants that had been “juvenilized” by repeated prunings. Similarly, Rathore et al. (57) used plant material for *in vitro* culture from pruned *Citrus limon* and a number of Indian woody species that are important biomass and pharmaceutical producers, such as *Balanites aegyptiaca* and *Syzygium cuminii*.

### 2.4. Forcing New Growth to Obtain Suitable Explant Material

A forcing solution system developed in Read’s laboratory has often been utilized to obtain soft new growth from existing buds which can be used for *in vitro* culture throughout the year (52, 58, 59). This system was adapted from methods used to enhance cut flower longevity (60) and involves immersing the bases of cut woody stems in solutions containing 2% sucrose and 8-hydroxyquinoline citrate at 200 mg/L; the resulting outgrowth of soft new shoots could then be used as softwood cuttings of sources of explants for *in vitro* culture (see Fig. 2). Although this forcing solution system did not result in rejuvenation, it was shown to be a reliable method for delivering plant growth regulating chemicals to the shoots serving as explant materials—for example, GA<sub>3</sub> to stimulate bud break and cytokinins and/or auxins to cause organogenic responses—and thus influencing *in vitro* performance.



Fig. 2. Forcing solution technology illustrated by *Aesculus* spp. stems in a vessel containing a solution of 2% sucrose and 200 ppm 8-hydroxyquinoline citrate (*below*) and examples of chestnut (*Castanea* spp.) hybrids produced *in vitro* from explants taken from similarly forced stems (*above*).

### **2.5. Forcing to Take Advantage of Epicormic Buds**

Harmer (61) demonstrated that isolated trunk sections of *Q. robur* placed in moist warm nursery conditions could produce shoots from epicormic buds. He also showed that girdling of intact trees could stimulate the growth of shoots from epicormic buds during the following growing season. Subsequent research with *Betula* illustrated that shoots from epicormic buds could be readily propagated *in vitro* (62). They observed that micropropagated shoots

exhibited morphology similar to seedlings, leading them to suggest that *in vitro* culture of paper birch may partially induce a change to a juvenile state. Cameron and Sani (63) produced shoots from epicormic buds from small logs of 5-, 10-, and 30-year-old *Betula* trees. Henry and Preece (64) also produced shoots from dormant stem sections of *Acer* species and Preece et al. (65) and Preece and Read (66, 67) reported on forcing shoots from the epicormic buds on logs of several woody species. Similarly, morphogenesis in Sitka spruce buds was accomplished from epicormic shoots (68); shoots from epicormic buds of elite trees of *Eucalyptus maidenii* (69) were cultured *in vitro*. The use of epicormic buds to obtain shoots for micropropagation of recalcitrant species such as *Quercus alba*, *Quercus bicolor*, and *Quercus rubra* (70) and teak (*Tectona grandis*) greatly aided successful micropropagation (71).

## 2.6. Grafting and Micrografting

Grafting of mature plant parts onto juvenile plants, often seedlings, has been employed to obtain cuttings that have a propensity to root readily. This approach has also been exploited to provide explant material for *in vitro* propagation. For example, *Uapaca kirkiana*, a dioecious tree in the Euphorbiaceae has historically been difficult to propagate vegetatively, but lateral shoots excised from grafted trees led to successful micropropagation (72). Another interesting example is the recent report of rejuvenation of Spanish red cedar (*Cedrela odorata*) by grafting elite mature tree twigs onto 3-month-old juvenile trees; this approach had a positive effect on the micropropagation rate of explants from mature material (73). Carron et al. (74) suggested that propagation of rubber tree (*Hevea brasiliensis*) by the use of buds from rejuvenated clones was a “good compromise” between micropropagation and conventional bud grafting. The *in vitro*-derived rejuvenated clones were produced by various *in vitro* techniques, including somatic embryogenesis from anther cultures.

Early examples of grafting *in vitro* (“micrografting”) include the research in Murashige’s laboratory, where Pliego-Alfaro (75) used aseptically germinated seedlings as rootstocks for *in vitro* grafting mature lateral buds of avocado (*Persea americana*), which led to approximately 50% of resultant shoots exhibiting “restored” rooting competence. Huang et al. (76, 77) further demonstrated restoration of juvenile characteristics of mature *Citrus* and *Sequoia sempervirens* by repetitious *in vitro* grafting, including restored vigor and rooting ability. Amiri (78) reported what he termed “homoplastic” *in vitro* grafting techniques, application of a few drops of agar medium to the graft union for micrografting mature sweet cherry (*Prunus avium*) onto seedlings of sour cherry (*Prunus cerasus*). He noted that in spite of some nutrient disorders and problems of contamination, this was considered a promising technique for propagation of disease-free cherry.

### 2.7. Somatic Embryogenesis

Almost by definition, embryos are juvenile plants, since it is essentially impossible for embryos to perceive and invoke floral stimuli. Although somatic embryos (those that are produced *in vitro*) should probably be referred to as “embryoids” since they are not the result of fusion of gametes, for simplicity we use the term embryos as we discuss embryogenesis. Many examples exist wherein the initial explant was seeds or seed-derived callus, rather than from mature plants or plant parts. Kanwar et al. (79) used callus from hypocotyls and cotyledons of *Robinia pseudoacacia* to obtain callus from which cell suspensions were produced that could yield viable plantlets. Li et al. (80) employed zygotic embryos to obtain somatic embryos from Korean spruce (*Picea koraiensis*) that were regenerated into intact plants. Likewise Prakash and Gurumurthi (81) were able to stimulate embryogenesis in *Eucalyptus camaldulensis* using zygotic embryos and cotyledon explants, and demonstrated that only the very young (10-day-old) explants provided significant embryogenic potential. Dhekney et al. (82) also studied the importance of embryogenic culture age on ultimate success of regeneration and potential for transformation. Similarly, to obtain embryogenic chestnut (*Castanea sativa*) Corredoira et al. (83) employed mostly zygotic embryo material as explants. In some cases, embryogenic callus may arise from rejuvenated mature tissues (see Fig. 3). Furthermore, a number of reports have indicated the presence of gibberellins (84) or influence of chemicals such as ABA (85–88).

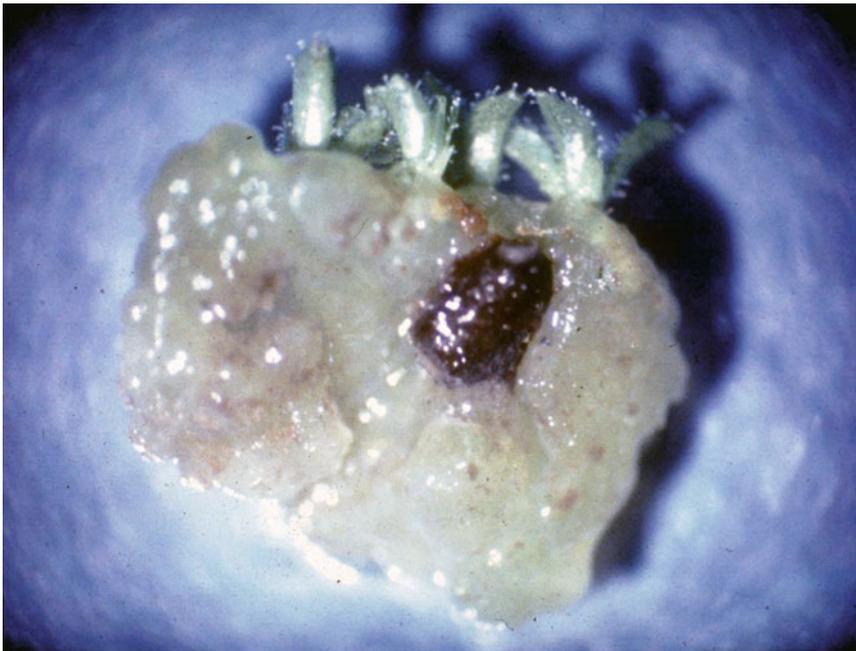


Fig. 3. Callus explants derived from rejuvenated internode segments (*dark oval*) of hybrid blueberry plants (*Vaccinium* spp.) exhibit significant competency for embryogenesis leading to shoot formation.

Krul and Worley (89) used a variety of explants from several grapevine cultivars to produce callus, but only “Seyval” produced embryoid-like protusions. They did not indicate which of their explant sources produced the embryogenic callus, but one could predict the likelihood of it being from the floral organ explants. Although these examples do not signify rejuvenation was achieved, Das et al. (90) developed an efficient method to achieve embryogenesis from grapevine leaf-disks cultured *in vitro*. In their work, when trying to germinate their embryos, cold temperature treatments were beneficial; this approach resembles the stratification necessary to germinate grape seeds, thus reinforcing the concept that embryogenesis is a type of rejuvenation. Serial grafting of *Kalopanax septemlobus* has been found to enhance somatic embryogenesis (91). They found that repeated grafting of shoots from mature *Kalopanax* trees led to rejuvenation of tissues that ultimately produced somatic embryos in a highly efficient manner.

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### 3. Conclusion and Prospects

It is readily apparent that *in vitro* culture has significant impact on phase change and specifically on rejuvenation. The simple act of micropropagation of explants obtained from mature plant tissue essentially causes rejuvenation by leading to the formation of microplants that exhibit one or more juvenile characteristics, the “juvenilization” referred to by Clapa and Fira (56) in relation to repeated pruning of stock plants. In some cases, rejuvenation may occur in the initial culture; in others, several subcultures may be required. Many horticultural manipulations have been taken advantage of to predispose explants to potential rejuvenation, including meristem culture; *ex vitro* and *in vitro* grafting; chemical treatments of stock (donor) plants and of explants; frequent hedging of stock plants; forcing new growth through a variety of means, especially epicormic buds; and the use of embryogenic and transgenic techniques.

Future applications of biotechnology are dependent on efficient micropropagation systems. Transformation by insertion of horticultural important genes has been achieved by parasexual hybridization (92), *Agrobacterium*-mediated transformation (93), and the use of biolistic transformation (the “gene gun”) (94, 95). Gray (96) and Gray and Meredith (97) have also suggested the potential for artificial seeds for grapevine transformation. Regardless of the technology employed, it is clear that *in vitro* rejuvenation will play a significant role in the growth and application of modern biotechnology (98).

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## Encapsulation of In Vitro-Derived Explants: An Innovative Tool for Nurseries

Alvaro Standardi and Maurizio Micheli

### Abstract

The encapsulation technology consists of the inclusion of some millimeter-long plant portions in a nutritive and protective matrix. This technology represents a further and promising tool for exchange of plant material between private and public plant tissue culture laboratories, for short- and medium-term storage of valuable plant material and for use of in vitro-derived or micropropagated propagules directly in farm or in nurseries. After encapsulation, transport, storage and sowing in aseptic conditions, the enclosed explants (*capsules*) may evolve in shoots (*regrowth*) and be employed for subsequent micropropagation or culture in vitro. When the encapsulated explant evolves in plantlet (*conversion*) in in vitro or in vivo conditions, the product of the encapsulation is defined as *synthetic seed* or *artificial seed* or *synseed*. The different evolution of the encapsulated plant material depends on tissue or plant material, genotype, nutritive and culture conditions, and treatments before or after encapsulation. In order to make economical the application of the encapsulation technology in the commercial nursery, research is looking for efficient automation or mechanization of the procedure and for preparation of the encapsulable explants.

**Key words:** Alginate bead, Capsule, Micropropagation, Synthetic seed

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### 1. Introduction

The nurserymen put all efforts to produce true-to-type and pathogens-free plant material, which is achieved by vegetative multiplication (or propagation) techniques and certification procedures, respectively. These tools are effective to assure high-quality of plants to the marketing systems. Since the world's demand for new and promising forestry, ornamental and horticultural genotypes is increasing, nursery operators are looking for research support and new strategies to produce plants by innovative and reliable effective technologies (1). Moreover, they are faced with intransigent regulations aimed to prevent the introduction of devastating pathogens with the infected plant material in most of the countries.

So, besides the traditional multiplication techniques such as grafting, cutting and layering, micropropagation is highly successful for large-scale plant production, maintaining their genetic fidelity and health. Nevertheless, large-scale deployment of the *in vitro*-derived plantlets implicates some practical problems during their management in the nursery. In fact, after the acclimatization, the plants are cultivated in the field, and face pests, pathogens and environmental and agronomic stresses, which are similarly faced by plants produced with traditional propagation methods. So, micropropagated plants can lose some advantages before the commercialization, precisely when the sanitary requirements have to be respected. In addition, the use of the micropropagated plants, which are not easy to manage, store, or transport and are exposed to the deterioration and damage risks, seems to have some commercial limits in comparison with the zygotic or gametic seeds, which combine advantages of reduced size, handling, storability and transportability (2), although they cannot be used for propagation of clones, especially if obtained by open pollination. Therefore, the research is looking for new technologies that are able to join the advantages of micropropagation, as high productive efficiency, perfect sanitary plant conditions, reduced space requirements, with the characteristics of the zygotic seed (3).

The encapsulation technology can integrate the micropropagation to reduce the incidence of the problems above reported. As below described in detail, two different products can be obtained from this technology: capsule and synthetic seed (Fig. 1).

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## 2. Encapsulation Technology: Concepts, Procedure, and Applications

The concept of the encapsulation technology was born to satisfy the requirement of somatic embryos protection during their transport and manipulation in nurseries or in farms. In fact, Murashige (4) gave the first definition of *synthetic seed* (or *artificial seed* or *synseed*) as “an encapsulated single somatic embryo.” This definition limited the synthetic seed manufacture to the use of somatic embryos encapsulated into a matrix which would allow their manipulation and sowing. Kozai et al. (5) reported the high risks of somaclonal variation associated with somatic embryogenesis, especially in some species, which represents a major problem, since it invalidates the basic rationale behind the synseed technology and the use of somatic embryogenesis as a clonal method for asexual plant multiplication (6). Bapat et al. (7) proposed the production of synthetic seeds also through the encapsulation of *in vitro*-derived propagules different from somatic embryos. The increasing number of studies focused on the use of nonembryogenic propagules for the manufacture of synthetic seeds allowed to extend their

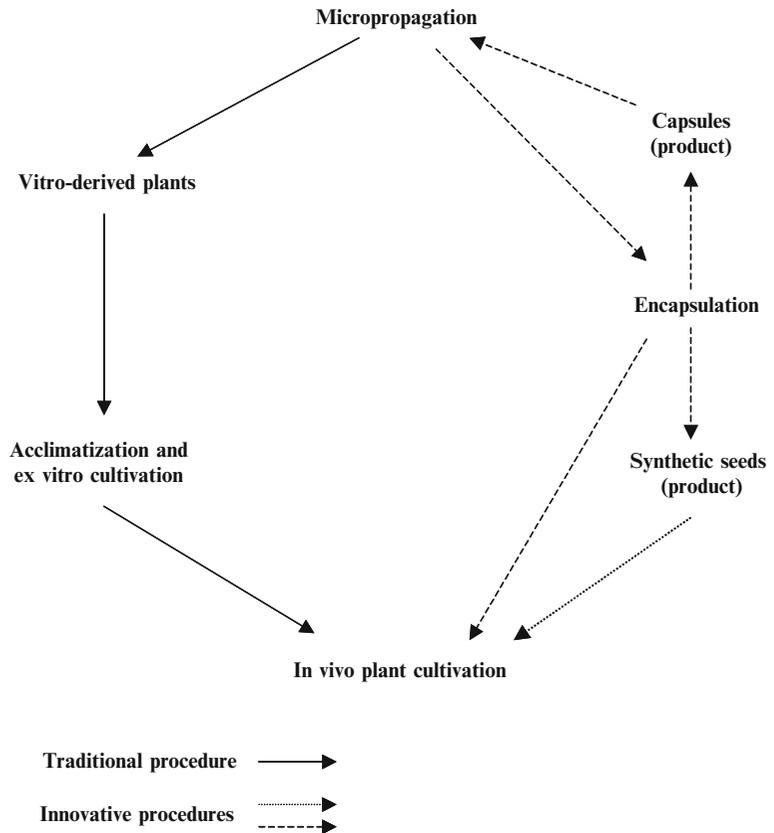


Fig. 1. Integration between the micropropagation (*left*) and the encapsulation technology (*right*).

definition as “artificially encapsulated somatic embryos, shoot buds or any other meristematic tissues used as functionally mimic seeds for sowing and possessing the ability to evolve into plantlets under in vitro or ex vitro conditions, which can be retained even also after storage” (8–11). Consequently, it is also possible to encapsulate directly meristems isolated from in vivo cultivated plants. Pattnaik et al. (12) excised buds directly from mature trees and used as synthetic seeds. Recently, Micheli et al. (13) and Standardi (14) proposed the *capsule*, a further product of the encapsulation technology, which can be defined as “an encapsulated portion of in vitro-derived plant tissue possessing the ability to evolve in shoot (not into a whole plantlet), which can be reused only for micropropagation after storage and/or transport”.

The procedure adopted to make capsules or synthetic seeds is the same and it includes three steps (Fig. 2):

1. *Coating*: it is a single process, carried out by inserting the propagule, excised from in vitro cultures, into a gelling or encapsulating solution for a few seconds. For this purpose,

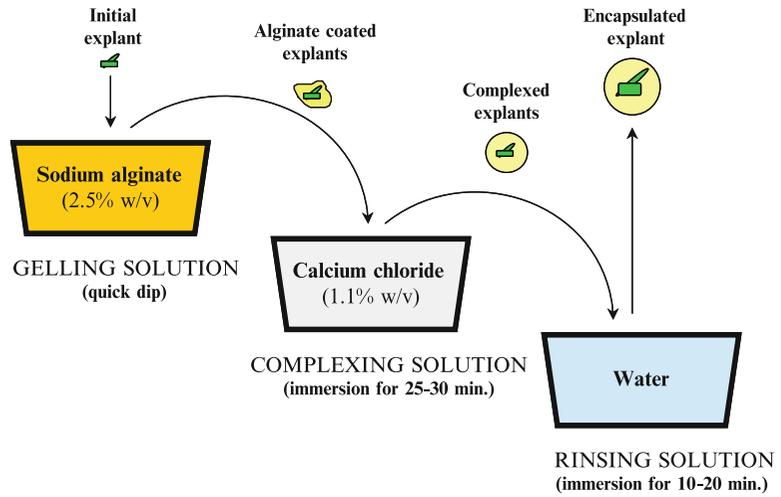


Fig. 2. The three steps of the encapsulation procedure adopted in our laboratory (University of Perugia, Italy).

sodium alginate is the most frequently used due to moderate viscosity, low spin ability of solution, no toxicity to the explants, quick gellation, low cost and biocompatibility characteristics. Moreover, it is largely employed because it provides better protection to the encapsulated explants against mechanical damages, depending on its concentration, level of viscosity or commercial type and from the complexation conditions. Nevertheless, many substances were essayed as encapsulating agents, in substitution to sodium alginate, like sodium alginate with gelatin, potassium alginate, polycy 2133, carboxymethyl cellulose, carrageenan, gelrite, guar gum, sodium pectate, tragacanth gum (11, 15, 16).

2. *Complexation*: it gives hardness to bead. The alginate-coated explants are dropped into a calcium chloride solution for 25–30 min. An ion-exchange process takes place during this period, resulting in the replacement of  $\text{Na}^+$  by  $\text{Ca}^{++}$  forming calcium alginate (10, 17). In detail, when the monovalent ion of sodium is replaced by divalent ions of calcium, ionic cross-linking among the carboxylic acid groups occurs, and the polysaccharide molecules form a polymeric structure called “egg-box” (18). So, the coating acquires the necessary consistence to assure protection against mechanical damages and dehydration risks. Hardening of calcium alginate bead is affected by the concentration of sodium alginate and calcium chloride and it may also vary in relation to the complexation time. Usually, higher texture corresponds to good protection during transport and manipulation, but higher difficulty to break the coating by the explant.

3. *Rinsing*: washing of complexed or coated explants in distilled water is required several times in order to remove the toxic residual ions of chloride and sodium.

After washing, such encapsulated propagules can be stored or transferred on sowing substrate (*synthetic seed*) or on proliferation medium (*capsule*). In any case, it is necessary that the encapsulated explants maintain their:

- *Viability*, i.e., green explants, with no necrosis or yellowing along the period between encapsulation and use.
- *Regrowth ability*, i.e., growth of explants with consequent breakage of the involucre and extrusion of at least one small shoot or root after sowing (for capsule and synthetic seed).
- *Conversion ability*, only for the synthetic seed (see below).

To satisfy these conditions, the two solutions employed for coating and complexation, besides the rinsing water, are enriched by nutrients and growth regulators (9). The composition of the nutritive solution can be similar to that employed for the *in vitro* proliferation phase over the micropropagation, but usually with all components at half concentration. This solution is currently called *artificial endosperm*, because it provides nutrition to the encapsulated explant, especially during its storage and regrowth: following the sowing, it mimics the role of the seed endosperm, besides adding to involucre the protective function (19, 20).

Some practical applications of both products of the encapsulation technology (capsules and synthetic seeds) are common and others are specific, as reported in Fig. 3. Moreover, this technology can be applied in research aimed to study different aspects on plant nutrition and plant physiology.

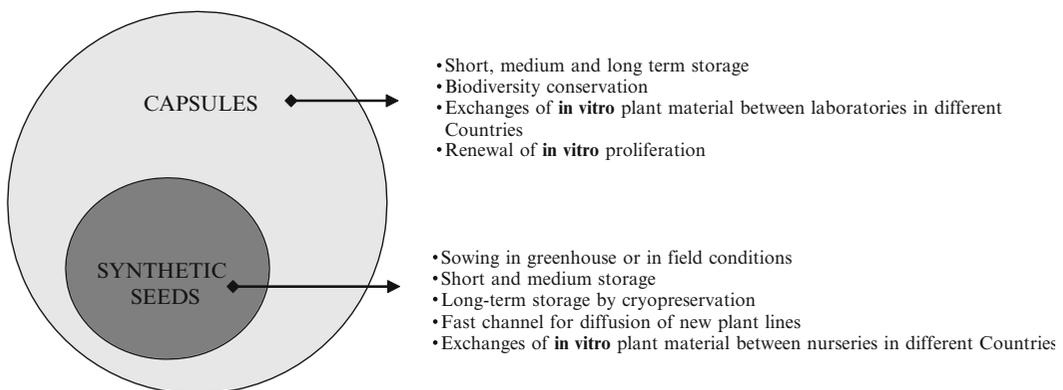


Fig. 3. Potential uses of products of the encapsulation technology.

### 3. Encapsulation for Synthetic Seeds

Redembaugh et al. (21) defined the term *conversion* used for somatic embryos as the contemporary growth of epigeous and hypogeous organs, with a vascular connection between shoots and roots, and reported that it corresponds to the term *germination* applied to the gametic or zygotic seeds. Afterwards Redembaugh (8) described the conversion as the production of a green plantlet from a synthetic seed, or in other words the development of a whole plantlet from encapsulated somatic embryos. Plantlets can be obtained also from unipolar encapsulated explants; therefore, the term conversion can be applied for all encapsulated plant tissues able to evolve in plantlets (8).

#### 3.1. Synthetic Seeds from Somatic Embryos

Somatic embryos are structurally similar to gametic or zygotic ones and possess many useful characteristics, including the ability to convert into plantlets, named “emblings,” while it is well known that the plantlets germinated from gametic seed are defined “seedlings” (16). However, unlike somatic embryos, the gametic or zygotic ones are the result of sexual processes and, in cross-pollinating species, the progeny is not genetically uniform and identical to one single parent. Somatic embryos develop from somatic cells and this regenerative pathway allows the clonal propagation. Furthermore, the somatic embryo lacks nutritive (endosperm) and protective (teguments or involucre) structures. Consequently, the application of somatic embryos is limited because of difficulties in handling, transport, maintenance of viability during long-term storage, and low vigor during conversion. Since by encapsulation somatic embryos are enclosed in a nutritive and protective covering, this technology represents an efficient solution to overcome the above-mentioned problems.

Many research groups have been working on somatic embryo encapsulation in different species including cereals, fruits, vegetables, ornamentals, medicinal plants, and forest trees. Currently, efficient protocols to produce and encapsulate somatic embryos are available in some species, e.g., alfalfa (22), banana (11), beech (23), celery (24), citrus (25–27) (Fig. 4), feijoa (28), guava (29–31), grape (32), mango (33, 34), papaya (35), pine (36), pistachio (37), oak (38), sandalwood (39), and other ornamentals, supporting the commercial application in the nursery, especially when applied to new or valuable genotypes (11, 40).

Nevertheless, the use of somatic embryos as encapsulating explants for synthetic seed production is limited because of involved risks, which are:

- Somaclonal variation.

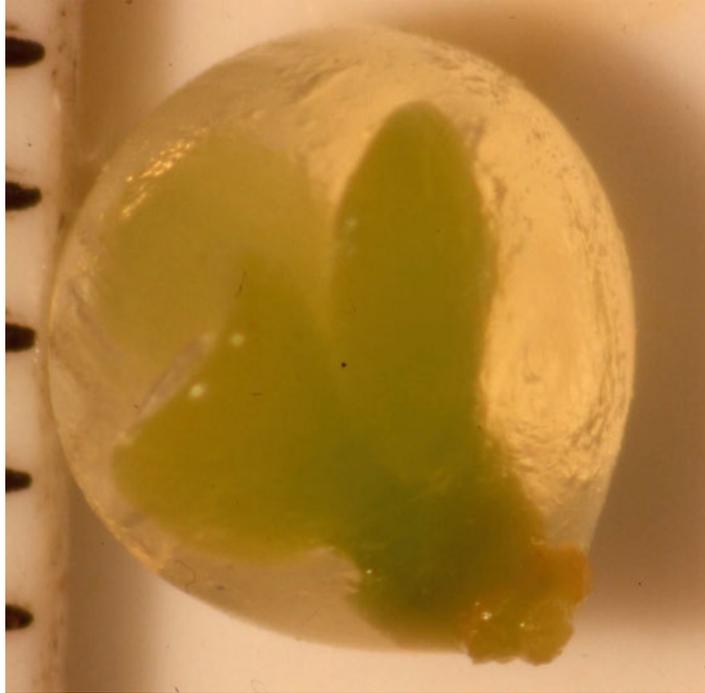


Fig. 4. Synthetic seed of *Citrus reticulata* Blanco, cv. Mandarino Tardivo di Ciaculli.

- Asynchronism during the somatic embryos formation and maturation.
- Recurrent embryogenesis (41).

However somatic embryos are able to be produced in bioreactors reducing their costs, making economically promising their practical use for the encapsulation technology (22).

While a number of research groups are looking for the solution of these problems, a new perspective in synthetic seed technology was opened with the use of nonembryogenic (unipolar) explants obtained by in vitro direct organogenesis or through axillary bud proliferation. In fact, reports on encapsulated microbulbs (*Lolium longiflorum*), epiphyllous buds (*Kalanchoe tubiflora*), protocorms (*Spathoglottis plicata*, *Vanilla planifolia*, *Cymbidium giganteum* and *Dendrobium wardianum*), rhizomes (*Nephrolepis* sp.), and hairy root fragments (*Armoracia rusticana*) were described and their conversion into plantlet was obtained without any particular inductive treatments (42–44). These organs are generally easy-to-obtain through direct organogenesis and risks of genetic variation are considerably reduced or absent (45, 46). However, it is important to consider that these promising propagules can be employed for the encapsulation technology in few species, whereas currently the use of unipolar in vitro proliferated explants is available in the majority of plant species to produce synthetic seeds.

### 3.2. Synthetic Seeds from Unipolar Propagules

The most frequently unipolar propagules used for encapsulation are 3–4 mm-long nodal portions of shoots with apical or axillary buds, excised during or at the end of the subculture; usually they are called *microcuttings* (Fig. 5). The absence of root primordia in these explants is often coupled with their inability to form adventitious roots spontaneously, which represents the major problem to obtain synthetic seed, whereas they are suitable to obtain capsules. Nevertheless, in some species, such as banana (47–49), *Cannabis* (50), *Dalbergia* (51), *Plumbago* (52), sandalwood (53), *Solanum* (54), *Valeriana* (55) and others (20, 56), encapsulated microcuttings demonstrated a high rooting ability after sowing, while numerous species did not perform as well, such as *Borivillianum* (57), *Dioscorea* (58), olive (59), *Phyllanthus* (60), pomegranate (61), *Spilanthes* (62), *Tylophora* (63), *Withania* (64), and many others (11, 65, 66).

Studies conducted on plant species difficult to rooting, as apple cultivars and rootstocks, olive, kiwifruit and peach, allowed to develop a procedure to induce rooting, in order to make the encapsulated microcuttings able to convert (9, 59, 67–69).

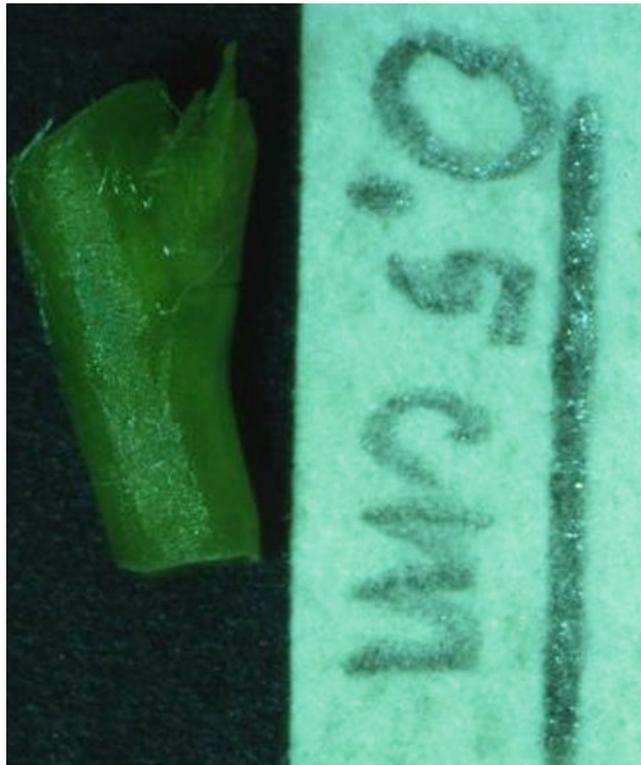


Fig. 5. Nodal microcutting of the M.26 apple rootstock, excised from an *in vitro* proliferated shoot.

This protocol, developed in the Laboratory of *in vitro* cultures of the Department of Agricultural and Environmental Sciences (University of Perugia), considers the following six phases (Fig. 6):

1. *Microcutting excision*: the shoots are separated from proliferated clumps at the end of the subculture and the apical and axillary portions, provided with one or two buds (according to species), are used to obtain 3–4 mm-long single-node microcuttings. The basal part of the proliferated clumps are always discarded, together with all the leaves and the oldest shoots and tissues. The microcuttings are characterized by different weight, 3–5 mg (*Rubus* sp.), and 13–15 mg (*Malus* spp.) according to the *in vitro habitus* of different species and cultivars (65).
2. *Root induction*: the microcuttings are transferred into 50-cc closed glass vessels (usually 10 per vessel) containing 15 mL of an inductive solution made of sucrose (15 g/L) and 1–5 mg/L indole butyric acid (IBA); the pH is usually adjusted to 5.5. The cultures are kept in darkness inside the growth chamber on a 100 rpm rotary shaker for 24–72 h, with the explants temporarily plunged in the solution.

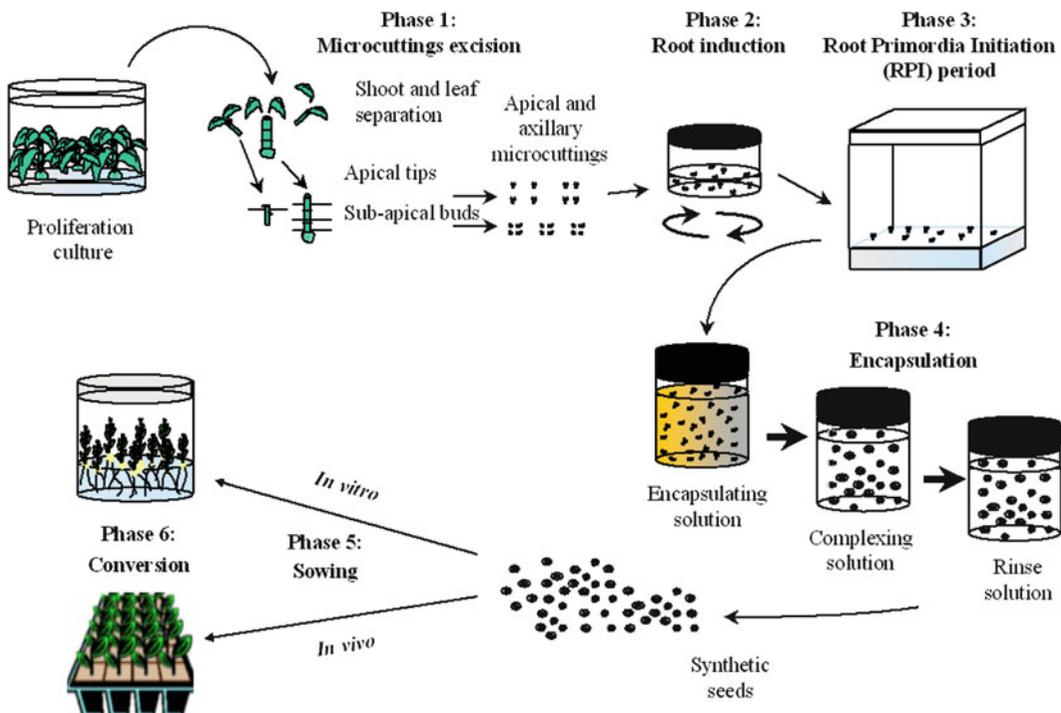


Fig. 6. Phases involved in synthetic seed formation using *in vitro*-derived unipolar microcuttings.

3. *Root primordia initiation*: after root induction, groups of five–ten microcuttings are placed in a Magenta® vessel (7×7×7 cm) containing 50 mL semi-solid proliferation medium (half-strength with 30 g/L sucrose) without growth regulators. In order to assure gas exchange between the explants and the environment, the microcutting are not immersed in the agarized (7%; w/v) medium, but placed on a sterile filter paper (Whatman #1), previously laid flat on the medium surface. The vessels are kept in the darkness, inside the growth chamber at  $21 \pm 2^\circ\text{C}$  for 6 days.
4. *Encapsulation*: the microcuttings are individually used for encapsulation procedure in calcium-sodium alginate beads, enriched with the artificial endosperm, adopting the steps previously described to obtain synthetic seeds (Fig. 7).
5. *Sowing*: after possible storage, synthetic seeds are sown in vitro or in vivo (ex vitro) conditions, inserting them just for some millimeters into the substrate, in order to avoid asphyxia of living tissues. Agar (6–8%; w/v), soil mix as Compo-cactea®, Gerbox™, Jiffy®, Milcap®, perlite, vermiculite, paper bridge, cotton, peat-sand mixture, garden soil, or others similar to natural conditions for the plant cultivation can be employed as



Fig. 7. Encapsulated apical microcutting of apple after rooting treatments (phases 2 and 3).

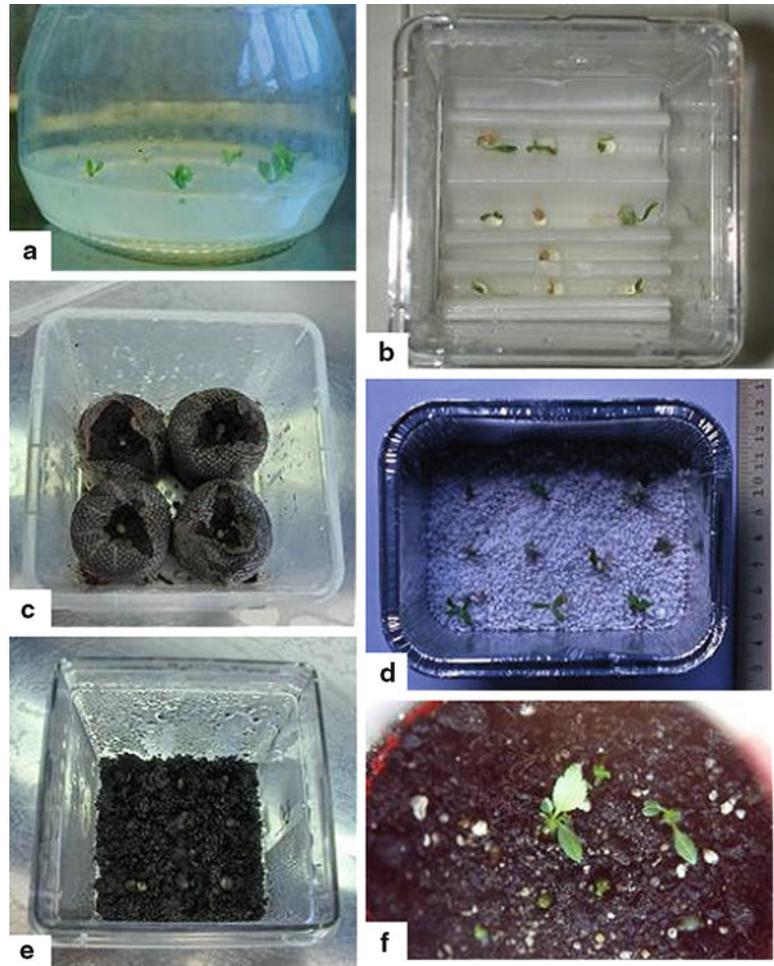


Fig. 8. Synthetic seeds, made by microcuttings, during conversion on agar (a), paper bridge (b), Jiffy-7® (c), perlite (d), Compo-Cactea® (e), and soil-mix (f).

sowing supports (11, 49, 70, 71). The sowing supports have to be enriched by nutritive formulation like the one employed for shoot proliferation, either reduced in the concentration or devoid of plant growth regulators, depending on the species (Fig. 8).

6. *Conversion*: usually this phase requires 4–6 weeks during which the cultures are maintained under growing conditions similar to micropropagation. After sowing of synthetic seeds, the developed plantlets (Fig. 9) are heterotrophic, requiring low light intensity and trophic support; gradually they become autotrophic and adequate nutritive and environmental conditions (light and temperature) have to be assured. Currently no reports, dealing with this specific aspect, are available.



Fig. 9. Olive plantlets, converted from synthetic seeds made by in vitro-derived microcuttings.

All phases are carried out in aseptic conditions, under a laminar flow cabinet, whereas strictly aseptic conditions can be avoided during conversion, especially when synthetic seeds will be finally transferred in field or into the nursery structures.

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#### 4. Encapsulation for Capsules

As previously reported and shown in Fig. 3, the *capsule* is one of the products of the encapsulation technology and it represents an effective tool for the exchange of elite and axenic plant material (germplasm) between laboratories due to its small size and relative easy handling (8, 33, 61, 65, 72, 73). In fact, plants produced by the traditional propagation methods pose severe quarantine problems because of their association with the transmission of pests and diseases. Moreover, in vivo and in vitro-derived plantlets are not easy to handle, store, and transport; they are always exposed to risk of deterioration and damage, thus limiting the exchange of genetic resources. So, an alternative tool for plant germplasm exchange nationally and internationally could be done by encapsulated plant material with an assurance of regrowth and proliferation abilities when re-subcultured for micropropagation (44, 57, 61, 73, 74). Up to now, the effects of the encapsulation and the artificial endosperm composition have been examined for microcuttings of several plant species (13, 20, 75, 76).

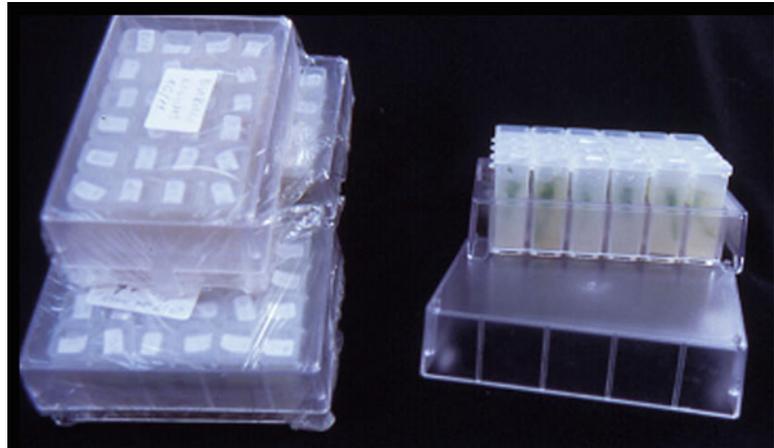


Fig. 10. Encapsulated microcuttings of olive stored in plastic cuvettes.

Moreover, the maintenance of capsules in sterile conditions and the avoidance of environmental stress during the commercialization period are important to assure the viability and the regrowth ability. One specific experience indicated that transferring the capsules in cuvettes made of semi transparent plastic material ( $1.5 \times 1.5 \times 4.5$  cm) represents an adequate procedure (Fig. 10). In each locked cuvette, ten capsules can be placed along with 1 mL of artificial endosperm solution to maintain the aseptic conditions and to avoid dehydration (77).

Another parameter influencing the viability and the regrowth is represented by the temperature and some of the above-mentioned researchers found that it is possible to store the capsules until 90 days in a common refrigerator at  $4^{\circ}\text{C}$  for a large number of genotypes. Moreover, in some species and for short period (until 30 days), the capsules can be stored at  $15\text{--}20^{\circ}\text{C}$  and in dark conditions.

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## 5. Encapsulation for Germplasm Conservation

The encapsulation could represent a supporting technology for plant material conservation, which can be carried out to allow two main goals:

- To elongate the commercialization period for short- or medium-term by low temperature storage ( $2\text{--}8^{\circ}\text{C}$ ).
- To maintain valuable genotypes for long-term by cryopreservation.

As for that, Rai et al. (11) reported that the conservation of encapsulated plant propagules can be achieved through ultra-low temperature using liquid nitrogen ( $-196^{\circ}\text{C}$ ) (78–80).

Several cryopreservation techniques, which include the encapsulation technology, are available, as encapsulation–dehydration and encapsulation–vitrification. In encapsulation–dehydration, the explants are:

- Encapsulated in alginate beads.
- Pre-grown in liquid medium enriched with sucrose for 1–7 days.
- Partially desiccated in the air current of a laminar air flow cabinet or with silica gel.
- Frozen rapidly (81).

This technique has been applied in several plant species, such as sugarcane, grapevine, apple, citrus, strawberry, and various ornamentals (82–85).

The encapsulation–vitrification is a combination between encapsulation–dehydration and vitrification, in which explants are:

- Encapsulated in alginate beads.
- Treated with cryoprotective substances (such as DMSO, glycerol, or PEGs).
- Dehydrated through vitrification solutions (e.g., PVS2; PVS3).
- Subjected to freezing (81).

This technique has been applied in strawberry, potato, cassava, and other plant species (84, 86, 87). Although the risks of somaclonal variation are reported, currently cryopreservation is routinely employed for long-term conservation of plant resources in a number of cases (81).

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## 6. Automation in the Encapsulation Technology

Though some steps of micropropagation could be carried out mechanically, at the moment obtaining synthetic seeds by microcuttings excised from proliferated shoots is relatively expensive due to:

- The shoot production from axillary buds during single subculture is a less-productive regenerative process compared to organogenesis or somatic embryogenesis.
- Excision of microcuttings from proliferated shoots requires time, specialized hand-labor, and care.

So, the application of this technology can be suggested only for high value genotypes and it is clear that the possibility of reducing the overall cost of producing and encapsulating microcuttings

through labor-saving processes or automation is a primary target (88). Since the mechanical excision of microcuttings suitable for encapsulation is not possible, automation protocol was tried for synthetic seed production; suitable explants for direct encapsulation were produced and subsequently regrowth by adventitious microshoot regeneration without any manipulation. Under this point of view, previous studies showed that encapsulable adventitious shoots of M.26 apple rootstock were achievable by direct organogenesis from leaves fragments (89) and some experiments were successfully carried out in different species (43, 90, 91). Using this different approach, a new protocol was proposed to automate at least some steps to obtain synthetic seeds with unipolar explants (Fig. 11).

Briefly, at the end of a proliferation subculture, the hand preparation of microcuttings is substituted by the mechanical fragmentation of all clumps and the derived explants are induced to produce adventitious shoots which are encapsulated as *microshoots* when their size ranges from 3 to 5 mm (92, 93).

Figure 12 summarizes the steps involved in the procedure to obtain encapsulable adventitious microshoots:

1. Proliferating cultures to obtain shoots for preparation of the organogenetic explants.
2. At the end of proliferation, basal callus is removed from the clumps, which are reduced in 3–4 mm portions by fast hand cutting, or grinding or mixing, according to species. During this procedure it is important to avoid that the machine (grinder or mixer) or the fast cutting creates stress to plant material: for this purpose the use of antioxidant solutions can be suitable. Moreover, Brischia et al. (92) found that the combination of IBA (0.1 mg/L), gibberellic acid (0.5 mg/L) and adenine hemisulphate (1 mg/L) was effective for adventitious shoot formation in M.26 apple rootstock.
3. To induce direct organogenesis, the fragments are transplanted in Petri dishes placed in the growth chamber for 15 days in darkness conditions, and for a further 15 days under the 16 h light photoperiod of  $40 \mu\text{mol}/\text{m}^2/\text{s}^1$  photon flux. Encapsulable adventitious microshoots are collected after 1 month from the beginning of the regenerative step. The size of the encapsulable explants is different, ranging from 3 to 5 mm, depending on the size and the type of the organogenetic fragments (93). Like in embryogenesis, also in organogenesis there is the inconvenience of asynchronism which can be overcome through the selection of microshoots by sieving or collecting them during organogenesis when a specific size range, experimentally predetermined, has been reached.
4. After harvesting, the encapsulable microshoots are submitted to the root-induction treatment, culturing them inside 50-cc closed jars containing 15 mL of solution made of 5 mg/L IBA

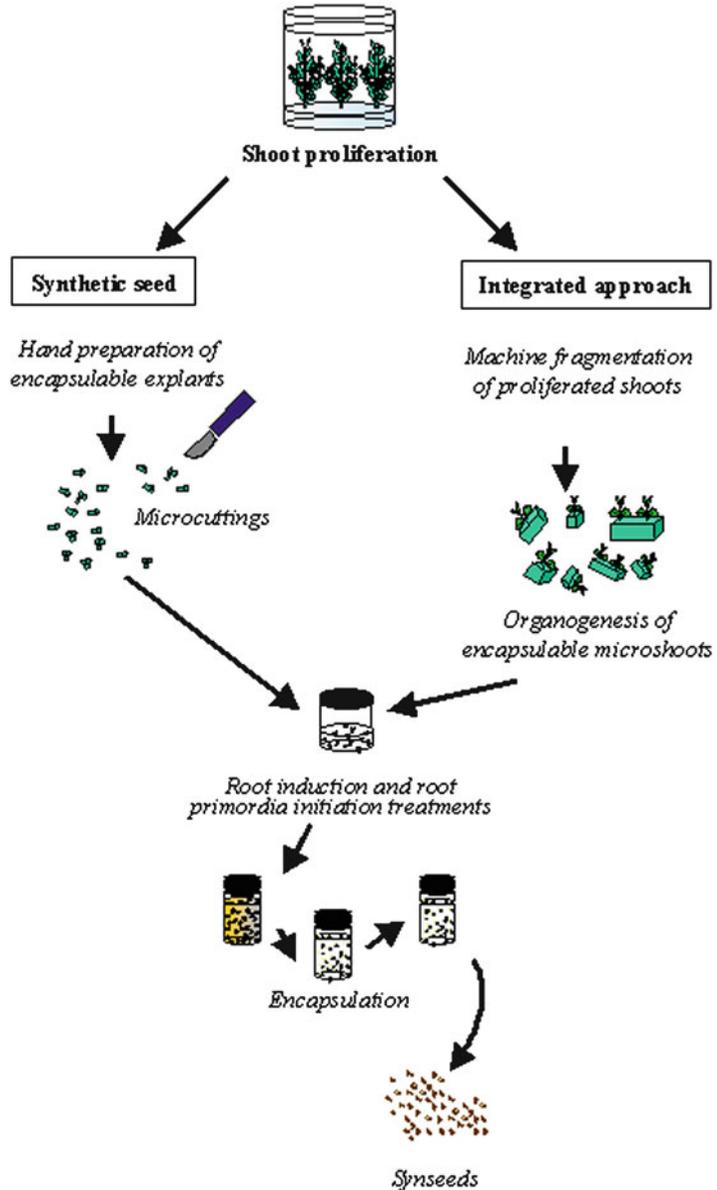


Fig. 11. Scheme to obtain encapsulable explants by hand excision (*left*) or machine fragmentation (*right*).

and 15 g/L sucrose at pH 5.5 (94). The jars are placed in darkness, inside the growth chamber at  $21 \pm 2^\circ\text{C}$  and shaken at 100 rpm for 24 h. Subsequently, the explants are placed inside 500-cc glass jars on filter paper (Whatman #1), laid flat on 100 mL of half-strength proliferation medium with 0.7% agar and without growth regulators, to optimize the gas exchange for root primordia differentiation. The jars are kept inside the same growth chamber for 6 days in the darkness.

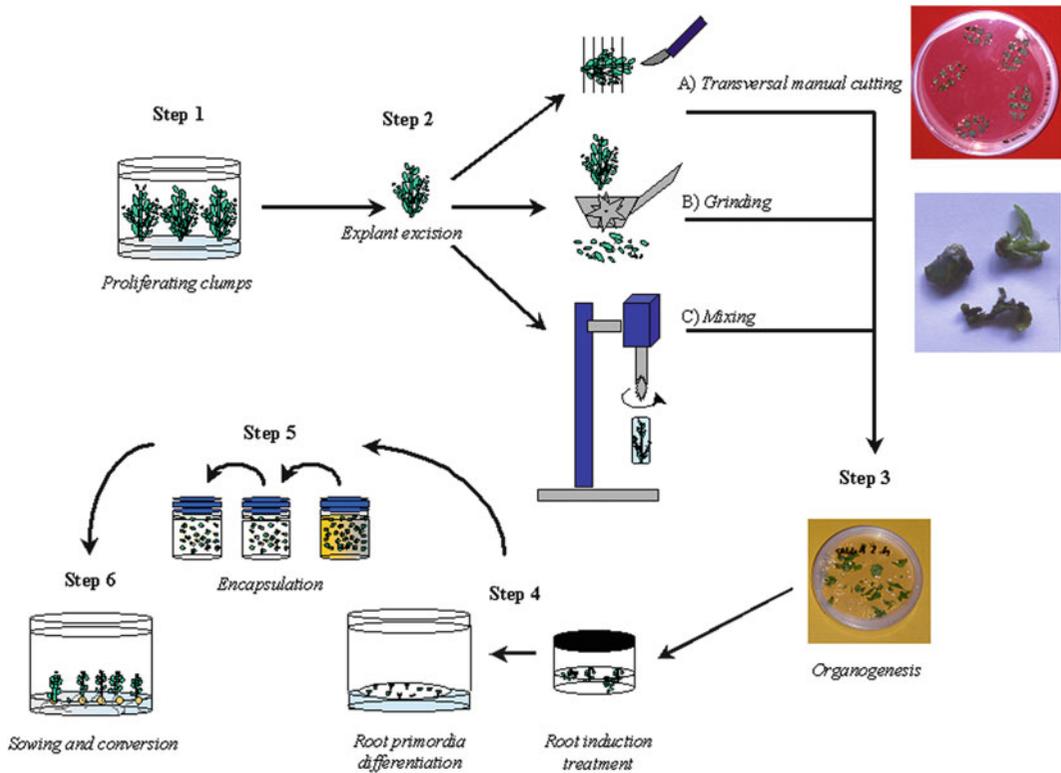


Fig. 12. Mechanical preparation of organogenetic explants and procedure for encapsulation of adventitious microshoots.

5. After this phase, the microshoots are encapsulated using the protocol already described.
6. Sowing of the synthetic seeds is performed as described above.

Because the encapsulation is a laborious procedure, mechanical techniques for automating the alginate coating are available, opening new opportunities for reducing the high costs currently connected with the commercial application of the encapsulation technology. Machinery for encapsulating plant propagules have to perform three functions:

- To isolate individual propagules.
- To insert each propagule into a separate alginate drop.
- To ensure the effective encapsulation of each propagule.

Several devices have been reported for the encapsulation of somatic embryos or other *in vitro*-derived vegetative propagules, using systems based on concentric tube nozzle, multiple wire loops, rotating disc, perforated plate, or precision dripping (92, 95–98).

## 7. Problems and Perspectives

The effort made on the development of encapsulation technology during these last three decades has made considerable progress. It has offered to nurseries an integrated package, including micropropagation for the production, commercialization, and exchange and conservation of germplasm.

Some problems can limit the large application of the encapsulation technology for synthetic seed production, demanding to the research effective solutions about: (a) the extrusion of the encapsulated explants from the alginate coating, lowering the alginate dosage, although this implicates leaching and dehydration problems and affects the gas exchanges of the enclosed explants; (b) the poor conversion in *in vitro* and *ex vitro* condition, studying the introduction of specific Arbuscular Mycorrhizal Fungi (AMF) inside the synthetic seeds (*biotization*) (99); (c) the control of fungal and bacterial contamination in non aseptic environment, verifying the effect of treatments to the sowing substrate or to synthetic seeds with specific products (fungicides, bactericides, or other antimicrobials) and the validity of biotization through AMFs and Plant Growth Promoting Bacteria (PGPB) increasing higher vigor and growth of plantlets with consequent reduction of encapsulated propagules loss (100, 101); (d) the leaching of nutrients and high dehydration risks, adopting the re-inclusion of the encapsulated explants into a new alginate coating procedure (double encapsulation) or covering the alginate bead with an impermeable pellicle, although the last proposal could determine asphyxia to encapsulated propagules (102); (e) the reduction of the manual labor to prepare the encapsulating explants and to carry out the encapsulation, studying automatic or semi-automatic tools and procedures.

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## Thermotherapy, Chemotherapy, and Meristem Culture in Banana

Ludivine Lassois, Philippe Lepoivre, Rony Swennen, Ines van den Houwe, and Bart Panis

### Abstract

Bananas that provide a staple food to the millions of people are adversely affected by several viruses such as Banana bunchy Top Virus (BBTV), Banana Streak Virus (BSV), and Cucumber Mosaic Virus (CMV). These viruses are known to have a devastating effect on crop production and constraint to the international exchange and conservation of banana germplasm—a cornerstone for breeding new cultivars. The viruses are particularly problematic in vegetative propagated crops, like bananas, because of their transmission in the planting material. Different virus eradication techniques have been developed, such as thermotherapy, chemotherapy, and meristem culture for providing virus-free planting material. Meristem culture proved to be the most effective procedure to eradicate phloem-associated viruses. This method requires isolation of meristematic dome of plant under the aseptic conditions and culture in an appropriate nutrient medium to develop new virus-free plants. Thermotherapy is another widely used virus eradication technique, which is initially carried out on in vivo or *in vitro* plants and eventually combined with meristem culture technique. The plantlets are initially grown at 28°C day temperature and increase it by 2°C per day until reaches 40°C and the night temperature at 28°C; maintain plants at 40°C for 4 weeks; excise meristem and culture onto the regeneration medium. In chemotherapy technique, antiviral chemical compound Virazole® is applied on meristem culture. Combination of these techniques is also applied to improve the eradication rate.

**Key words:** *Musa*, Virus eradication, Vegetative propagated plant, Tissue culture

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### 1. Introduction

Viruses have most devastating effect on crop production. They are a major problem in vegetative propagated crops, such as bananas. The viruses transmit in the planting material during vegetative propagation using suckers or during *in vitro* multiplication. The availability of virus-free planting material is highly dependent on the availability of efficient virus eradication techniques. Banana is severely affected by several viruses such as Banana Bunchy Top

Virus (BBTV), Banana Streak Virus (BSV), and Cucumber Mosaic Virus (CMV). Different virus eradication protocols were established, such as thermotherapy, chemotherapy, and meristem culture. The combination of these techniques is also applied to improve the eradication rate.

The meristem culture is based on the observation, frequently made in virus–host combination studies showing that the meristem often remains virus-free. Different researchers have shown that the rate of obtaining a virus-free regenerated plant is inversely related to the size of the isolated meristem (1). However, the capacity of the meristem to regenerate into a full plant is also directly related to the size of the explant. Hence, a balance between successful virus elimination and the probability for plant development is needed. Thermotherapy is another widely used virus eradication technique that can be carried out on *in vivo* or *in vitro* plants and which eventually could be combined with meristem culture. Relatively little is known about the mode of action of thermotherapy despite its widespread use. This technique is highly effective in virus eradication rates, which depends on the virus type. Moreover, it is time-consuming and *in vitro* plant material shows high mortality rate. The third virus eradication technique is chemotherapy, which uses chemical compounds applied to *in vitro* plant or meristem cultures. A wide range of potentially antiviral molecules has been tested. All of them have shown phytotoxicity and negative effects on meristem growth (2–6). Thus, it is necessary to test varying concentrations and treatment time to establish an optimal balance between the virus elimination rate and the plant survival rate. Among the antiviral substances tested, Virazole® (Ribavirin, 1-B-D-ribofuranosyl-1-2-4-triazole-3 carboxamide), a synthetic analogue of guanosine, is the most frequently used. Virazole acts on virus synthesis rather than through a direct inactivation of the existing virus. It is thus necessary to apply virazole for extended periods of time to eradicate viruses from infected tissues. During the treatment, virazole acts directly or indirectly preventing synthesis of new virus particles while existing virus particles are degraded in the course of their ontogeny (7).

Meristem culture is the most effective procedure for the eradication of phloem-associated viruses. Indeed, virus particles located in the phloem probably cannot invade the meristematic tissues because there is no cell differentiation in this zone. In case of BBTV, known to be phloem-associated (8), meristem culture from *in vitro* banana (*Musa acuminata*, AAA, cv Williams) plants turned out to be very efficient, resulting in 99% disease-free plants. The eradication rate was only 4% when meristems were excised from *in vivo*-grown plants. For BSV, eradication rates after meristem culture reached 76% and 41% by excising meristems from *in vivo*- or *in vitro*-grown banana plants, respectively (*Musa acuminata*, AAA, cv Williams). Thermotherapy combined with meristem

culture slightly increased the number of BSV-free plants up to 79% and 67%, respectively, when meristems were excised from heat-treated *in vivo*- and *in vitro*-grown plants (Helliot, unpublished results). The successful treatment of BSV-infected plants with three different acyclic nucleoside phosphonate analogues was also reported (6). With reference to CMV eradication, meristem culture alone is not very effective since only 1% and 7% plants were virus-free with the excision of meristems from *in vivo*- and *in vitro*-grown plants, respectively (9). This low eradication efficiency could be due to the localization of CMV particles within the banana meristematic dome (10). However, the CMV eradication frequency could be enhanced with more severe treatments. CMV eradication was achieved by Gupta (11) in approximately 100% regenerated plants by using meristem culture in combination with a 2-week heat therapy (38–40°C). Helliot et al. (9) obtained an eradication frequency 38% by excising meristems from *in vivo*-grown plants, and 70% from meristems—sourced from *in vitro*-grown plants, both combined with thermotherapy. Despite a diurnal alternating heat treatment, 60% mortality was still seen among the *in vitro*-grown plants, while *in vivo* plant material showed less sensitivity (4% mortality). The CMV eradication rate on banana, obtained with virazole chemotherapy, reached 24% (9). Previously, Long and Cassels (12) obtained 100% elimination of CMV on tobacco explant cultures treated with virazole.

All these results demonstrate that the virus eradication technique to be applied depends on (a) the virus characteristics, (b) the type of tissues treated, and (c) the plant species. Furthermore, our results concluded that banana genotype is the fourth major factor influencing the efficiency of a virus eradication technique.

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## 2. Materials

### 2.1. Instruments and *In Vivo* Plant Material

1. Sterile distilled water.
2. Ethanol 70% (v/v).
3. Sodium hypochlorite (NaClO, 5%).
4. Dissecting instruments: scalpels with disposable blades and forceps.
5. Sterile plasticware (e.g., Petri dish) or glassware.
6. Laminar air-flow cabinet.
7. Flame to sterilize instruments or glass bead sterilizer.

### 2.2. Culture Media

1. Media based on the formulation of Murashige and Skoog (MS; (13)) (Duchefa, Haarlem, The Netherlands) (see Note 1).
2. Sucrose as carbon source.

3. Indole-3-acetic acid (IAA) as auxin.
4. 6-Benzylaminopurine (BAP) as cytokinin.
5. Ascorbic acid as antioxidant.
6. Gelrite® or another comparable solidifying agent.
7. Sterile distilled water.
8. Concentrated NaOH (1 M).
9. Concentrated HCl (1 M).
10. pH-meter.
11. Analytical (Ohaus-Adventurer) and precision balance (Sartorius).
12. Hotplate/stirrer.
13. Refrigerator to store prepared media, stock solutions, and some chemicals.
14. Autoclave.
15. Aluminum foil.
16. Screw-capped glass test tubes, 50–100 mL capacity.
17. Tube holders (metallic or plastic racks).
18. For chemotherapy:
  - Virazole® (Ribavirin, 1-B-D-ribofuranosyl-1-2-4-triazole-3 carboxamide; Duchefa, Haarlem, The Netherlands).
  - Syringe with syringe filter unit, pore size 0.2 µm (Acrodisc, VWR, Belgium).

**2.3. Meristem Culture, Thermotherapy, and Chemotherapy Combined to Meristem Culture**

1. Laminar air-flow cabinet.
2. Dissecting instruments: scalpel with disposable blades (min. two sizes) and forceps (min. two sizes, a longer round tipped and a short fine tipped).
3. Ethanol 70%.
4. Flame to sterilize instruments or glass bead sterilizer.
5. Petri dishes (Ø 90 mm).
6. Parafilm.
7. Binocular microscope with light source.
8. Culture room.
9. Incubator.

**2.4. Plant Acclimatization in the Greenhouse**

1. Tap water.
2. Greenhouse.
3. Clear plastic for tunnel.
4. Vermiculite.
5. Non-sterilized soil.
6. Plant pots (from Ø 7 cm).

### 3. Methods

Among virus eradication protocols (meristem culture, thermotherapy, and chemotherapy), the selection of the most appropriate procedure is made according to the virus type (BBTV, CMV, or BSV). However, eradication efficiency varies depending on the banana genotype and alternative methods may have to be tested when the reference treatment fails. Furthermore, particular attention should be given to BSV. This is due to the fact that the activation of specific integrated virus sequences is associated with some genome of cultivated *Musa* (14). Eradication of viral particles could be counter balanced by the activation of these integrated sequences induced by the stressful eradication treatment and the *in vitro* procedure. A schematic representation of banana virus eradication process is shown in Fig. 1.

#### 3.1. Aseptic Working Conditions

A critical step during the *in vitro* process is the maintenance of aseptic conditions and general precautions, presented hereafter, should be respected (see Note 2).

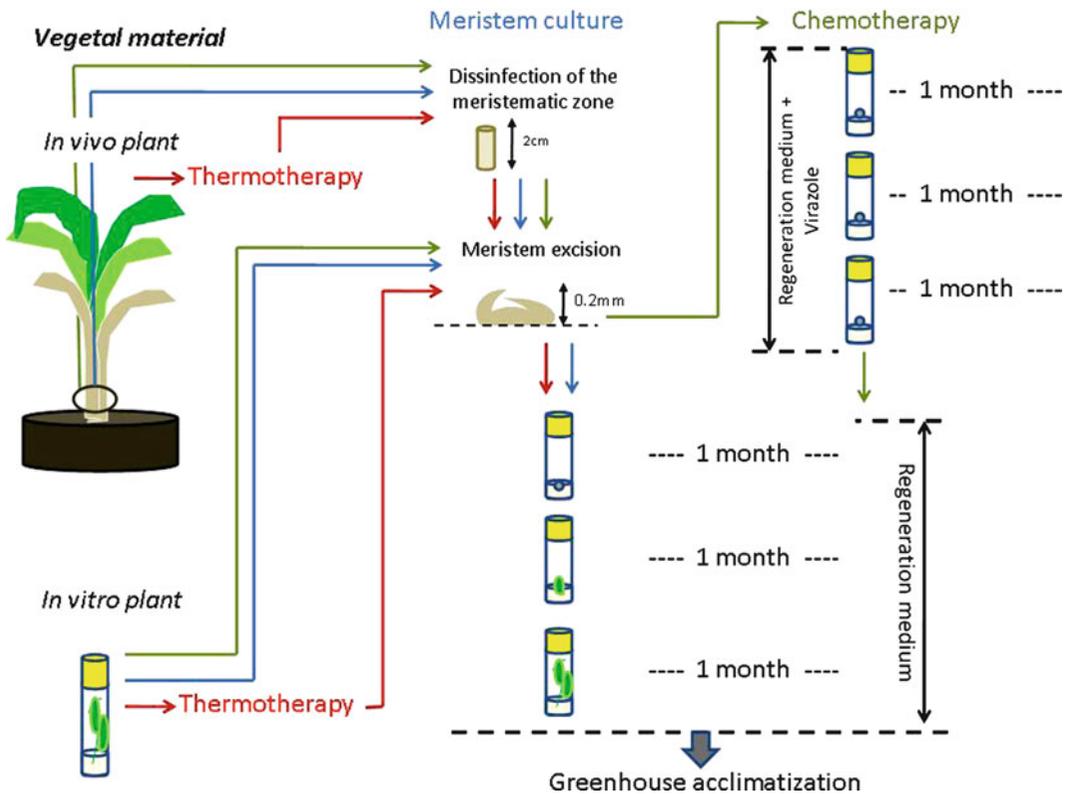


Fig. 1. Schematic representation of virus eradication processes for banana. The *green, blue, and red arrows* represent the process of chemotherapy, meristem culture, and thermotherapy, respectively.

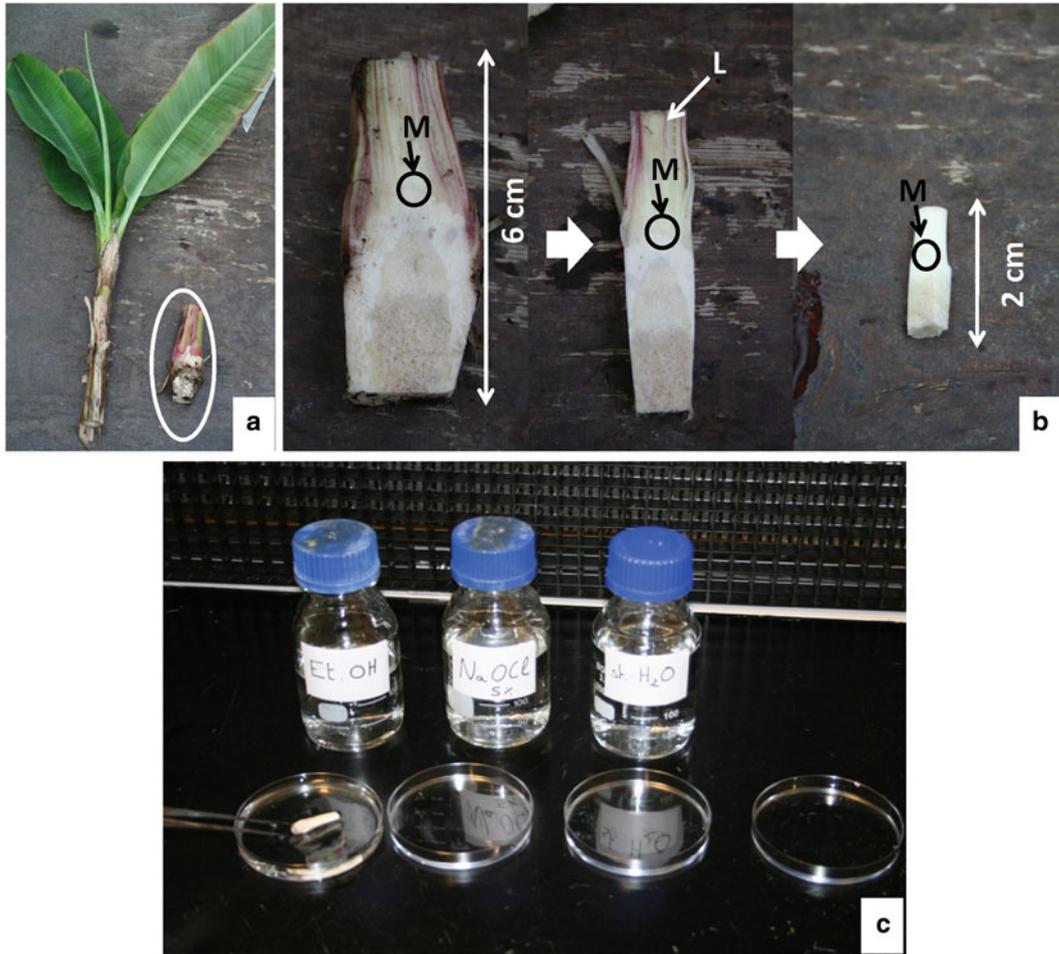


Fig. 2. Explant preparation and disinfection procedure for in vivo banana plants. (a) Separation of the meristematic zone from the pseudostem with a non-sterile knife; (b) successive trimming tissues to obtain a  $1 \times 1 \times 2$  cm cylinder, containing the meristematic zone; (c) disinfection of the cylinder (*M* meristematic zone localization; *L* successive leaf sheaths).

1. Regularly disinfect thoroughly operator hands with ethanol 70% or disinfecting soap.
2. Clean and disinfect the meristem transfer area and all the equipment needed in the meristem transfer area such as the binocular microscope, light source, etc., with ethanol 70%.
3. Use only sterile plasticware and glassware.
4. Use only sterilized media and products.
5. Autoclave dissecting instruments. They are repeatedly sterilized during manipulation, either by soaking in 70% ethanol followed by flaming or by using glass bead sterilizer if available.
6. Sterilized parts of instruments and plant material should not be touched by hands.

### 3.2. Culture Media Preparation

1. Fill a beaker with 800 mL distilled water.
2. Start heating and stirring.
3. Add powdered MS (including micro-, macro-elements, and vitamins) sourced from a commercial supplier (e.g., Duchefa, Haarlem, The Netherlands) (see Note 1).
4. Add plant growth regulators: for both regeneration and proliferation media, use 1 mL/L IAA and 1 mL/L BAP from stock solutions of  $10^{-3}$  M, stored at 4°C (growth regulator concentration in the medium: 1  $\mu$ M).
5. Add 1 mL/L ascorbic acid from a stock solution of 10 mg/mL, stored in darkness at 4°C, and packed in aluminum foil from no more than 1 month.
6. Add 3% (30 g/L) sucrose.
7. Add 3 g/L gelrite.
8. Add distilled water to a volume of 1 L.
9. Adjust pH to 5.8 using concentrated NaOH (1 M) or HCl (1 M).
10. Heat medium until a transparent solution is obtained.
11. Put 10–15 mL medium into each of the glass tubes.
12. Close the tubes with the caps, put them in tube holders (racks).
13. Sterilize by autoclaving at 120°C for 20 min.  
For chemotherapy, modify the previous protocol as follows:
14. Autoclave the bottle containing the medium just after the pH adjustment at 5.8.
15. Freshly prepare an antiviral stock solution by dissolving the antiviral molecule (virazole) in sterile distilled water at a final concentration of 10 mg/mL.
16. Leave the medium to cool to about 50°C and add the antiviral solution via filter sterilization with a syringe filter 0.2  $\mu$ m, under aseptic conditions, to obtain a final concentration of 50 mg/L.
17. Put 10–15 mL antiviral regeneration media in sterile glass tubes.

### 3.3. Preparation and Disinfection of *In Vivo* Material for Meristem Excision

Culturing meristems includes a process of surface sterilization when isolated from *in vivo* plants. This plant material might be contaminated with microorganisms which could grow very rapidly and kill the plant *in vitro* cultures. When the meristem is isolated from *in vitro*-grown plants, disinfection is not needed and excise meristem immediately under the aseptic conditions.

1. Select *in vivo*-grown plants.
2. Reduce the size of the pseudostem to the meristematic zone and surrounding tissue by cutting the plant with a sharp knife (see Note 3; Fig. 2a).

3. Remove superfluous tissue by successive trimming away the outer leaf sheaths, leaf bases, and corm tissue until obtaining a 1 × 1 × 2 cm cylinder, enclosing the meristematic zone (see Note 4; Fig. 2b).
4. Wash the tissue cube under tap water.  
Subsequent steps should be conducted in aseptic conditions:
5. Rinse the tissue cube by immersion (e.g., in a Petri dish) 70% ethanol for 2 min (Fig. 2c).
6. Wash the explants with sterile distilled water and put it in a solution of NaOCl 5% for 20 min.
7. Rinse the explants two times for 5 min and two times for 10 min with sterile distilled water.

The cubes of tissues are now ready for excision of meristems.

### **3.4. Culture of Meristem**

The meristem is the active growing point of the plant shoot. It is a small zone composed of dense, meristematic cells, which divide very fast. Meristem culture includes a process of tissues surface sterilization, the excision or isolation of the meristem, and its culture in a media under adequate conditions. The dome of the apical bud contains the real meristematic cells and is surrounded by leaf primordia and young leaves (Fig. 3). Isolation of the meristematic dome in aseptic conditions and its culture in an adequate aseptic nutrient medium leads to the development of plantlets. The meristematic cells divide and the differentiation of new tissues continues.

#### **3.4.1. Isolation of Meristems**

This step is delicate requiring patience, skill, and a lot of practice (see Note 5). Moreover, it is recognized that every meristem-explanting person develops his proper method of working. The whole processes (Fig. 4) should be performed in aseptic conditions as previously described (Fig. 5a).

1. Sterile instruments (Fig. 5b): minimum two scalpel holders with disposable blades and two forceps, and maintain their sterility.
2. Place the disinfected cylinder of tissue or the *in vitro* plantlets in a sterile Petri dish and hold it with the forceps (Fig. 5c).
3. Carefully remove the successive leaves, overlapping the meristem, by cutting with a scalpel through the circular insertion of each ones (Fig. 4). When the explant becomes too small, use a binocular microscope for a precise excision (see Note 4; Fig. 5c, d).

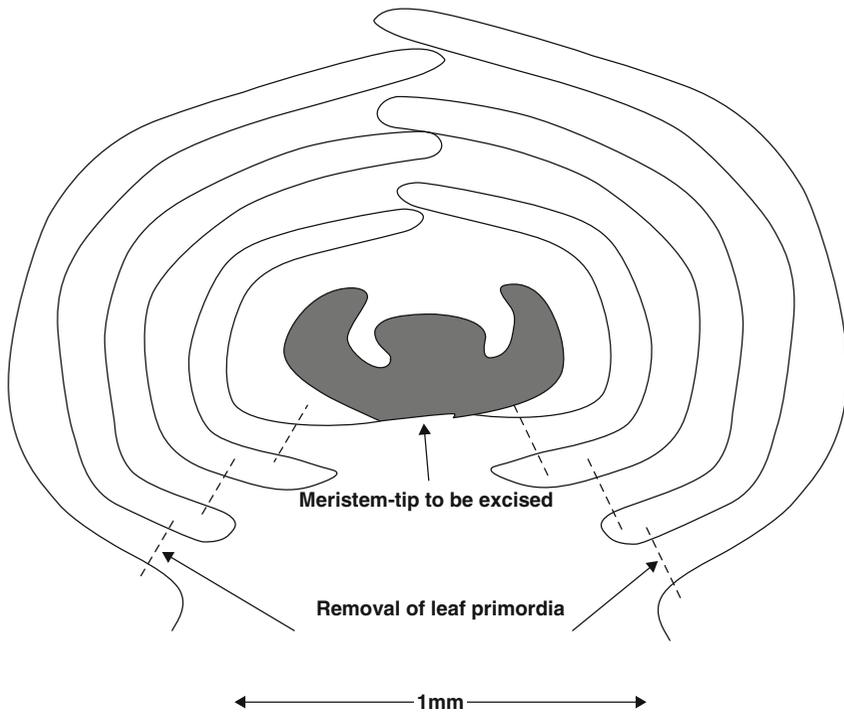


Fig. 3. Meristem-tip region.

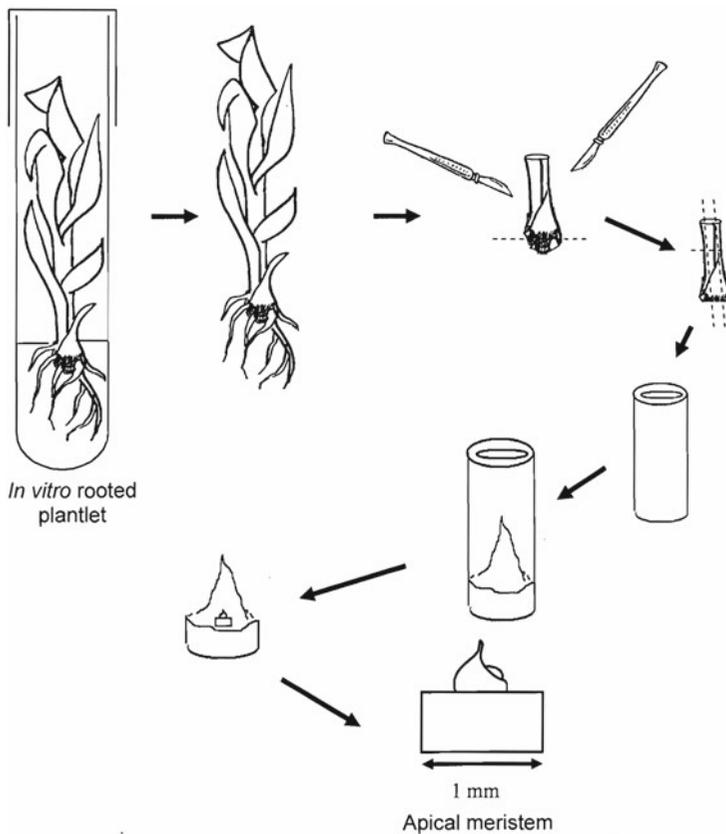


Fig. 4. Schematic representation of meristem excision from in vitro plantlets.

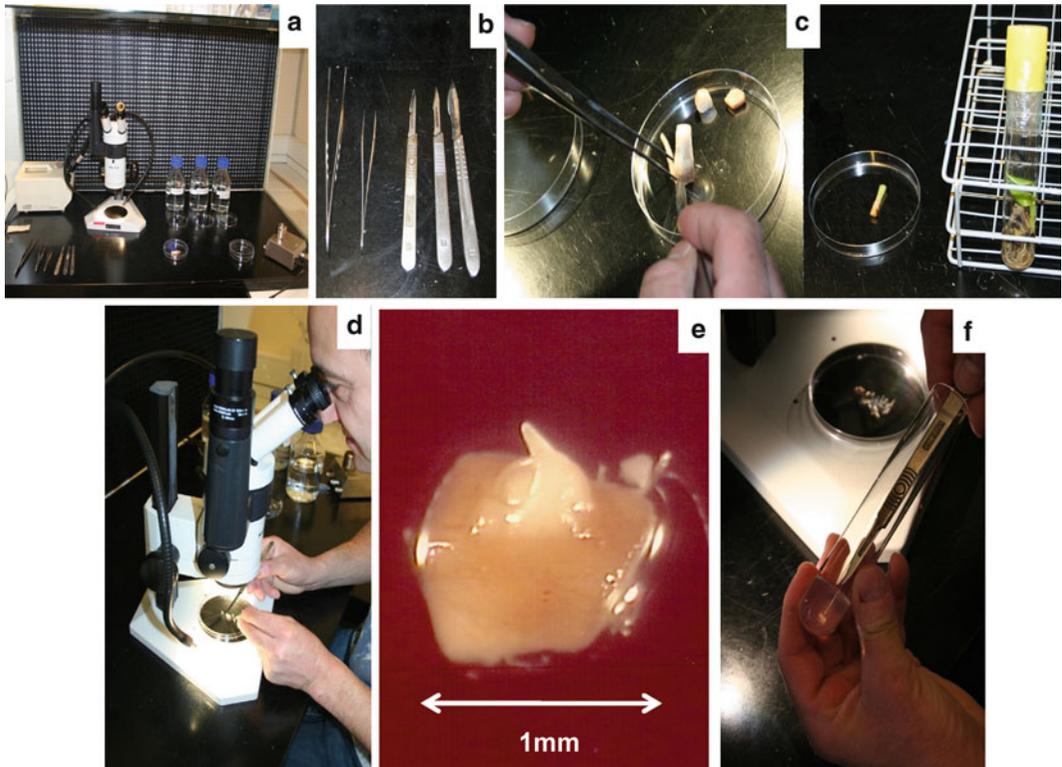


Fig. 5. Meristem culture. (a) equipment and supplies needed in the culture preparation area. From left back to right front: lamp, dissecting microscope binocular, ethanol with Petri dish, sodium hypochlorite with Petri dish, sterile water with Petri dish, dissecting tools, Petri dish containing explants from in vivo plant, Bunsen burner; (b) dissecting tools (forceps and scalpels of different size); (c) meristem excision from in vivo disinfected explant, or from an in vitro plantlet, using sterile forceps and scalpels; (d) meristem excision under a binocular microscope; (e) excised apical dome; (f) meristem tip transfer on sterilized medium.

4. Excise the apical meristem tip (< 1 mm diameter; Fig. 5e) consisting of the meristematic dome with 1–2 leaf primordia (see Note 6) with the second sterile scalpel (see Note 7).

#### 3.4.2. Meristem culture

1. Transfer immediately meristem tips from the scalpel to the glass tubes with screw caps (Fig. 5f); this operation is performed by sticking the scalpel in the sterilized culture media and slowly withdrawing it.
2. Cover the cap with parafilm.

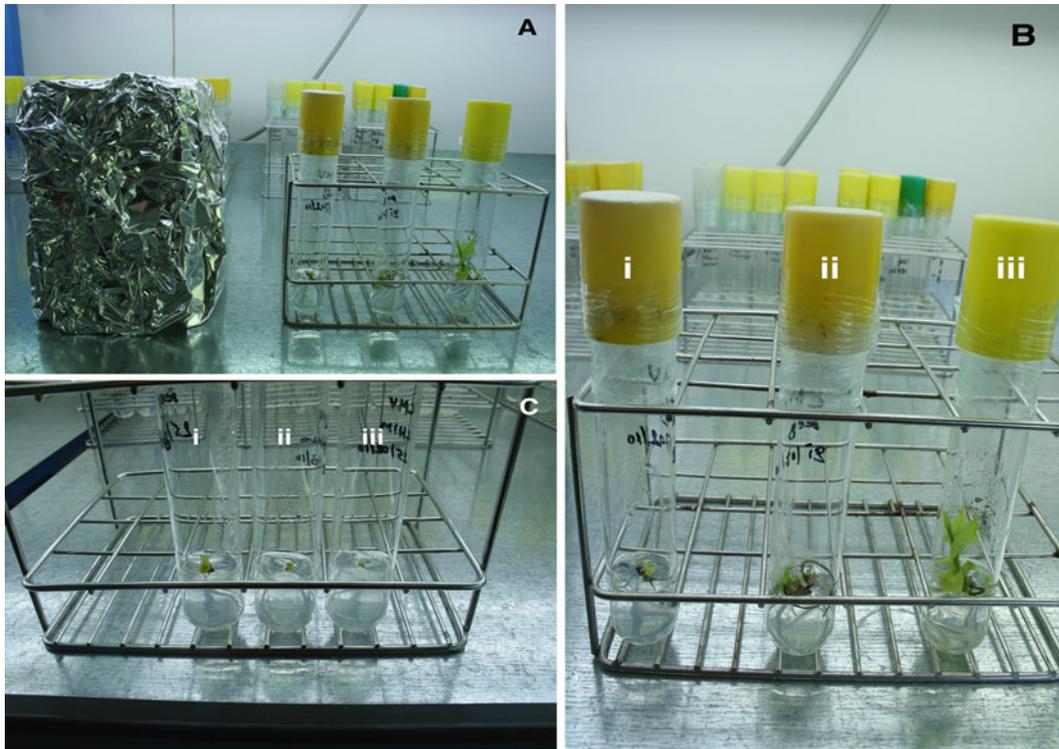


Fig. 6. Meristem cultures in the growth room. (a) Test tube rack covered with aluminum foil to obtain dark culture conditions, and without aluminum foil under a 16 h light/8 h dark cycle; (b) basket showing three tubes with banana meristem cultures at different stages of growth on the regeneration medium (i, after 1 month; ii, 2 months; iii, 3 months); (c) one-month-old cultures of banana meristems, excised from *in vitro* cultures subjected to a therapeutic treatment (i, after meristem culture only; ii, after thermotherapy; iii, after chemotherapy).

3. Place the tubes in a growth culture room at  $24 \pm 1^\circ\text{C}$  in dark conditions (see Notes 8 and 9) for 7 days, followed by a 16 h light/8 h dark cycle for 24 h (see Note 10; Fig. 6a).
4. During minimum 3 months (see Note 11), transfer monthly the dissected meristem to a new glass tube containing sterilized fresh culture media with sterile forceps (see Note 12; Fig. 6b/i,ii).
5. When plantlets have two or three leaves and some developed roots (Fig. 6b/iii), they can be multiplied on a proliferation culture medium or acclimated in the greenhouse.

### 3.5. Chemotherapy Combined to Meristem Culture

All these steps are performed in aseptic conditions.

1. Excise the meristem and transfer into the screw-capped glass tubes containing the antiviral regeneration medium (Fig. 6c).
2. Cover the cap with parafilm.
3. Place tubes in a growth cabinet (culture room) at  $24 \pm 1^\circ\text{C}$  in darkness for 7 days, followed by a 16 h light/8 h dark cycle per 24 h.

4. Using sterile forceps, transfer monthly for three times the dissected meristem to new glass tubes, containing sterilized antiviral regeneration media (see Note 13).
5. Transfer plantlets to fresh regeneration media, free of the antiviral compound, to allow their growth.
6. Transfer plantlets monthly to new glass tubes, containing sterile regeneration media.
7. When plantlets have two or three leaves and well-developed roots, they can be multiplied on a proliferation culture medium or acclimated in the greenhouse.

### **3.6. Thermotherapy Combined with Meristem Culture**

1. Place *in vivo* or *in vitro* plantlets in a growth cabinet (culture room) under a 16 h light/8 h dark cycle at 28°C.
2. Allow the temperature to increase by 2°C per day from 28 ± 1°C to 40 ± 1°C during the 16 h light period. Maintain always the temperature of the 8 h dark period at 28 ± 1°C.
3. Once 40 ± 1°C is reached, leave the plant material at this daily temperature (40 ± 1°C during 16 h of light, and 28 ± 1°C during 8 h in the dark) for 4 weeks.
4. Excise the meristem.
5. Transfer the meristem onto regeneration medium (Fig. 6c).
6. When plantlets have two or three leaves and well-developed roots, they can be multiplied on a proliferation culture medium or acclimated in the greenhouse.

### **3.7. Plant Acclimatization**

1. Remove plantlets from the glass tubes. Gently wash with tap water the adhering agar from the roots, paying attention to avoid any damage.
2. If more than one plant is growing in the tube, separate them into individual plant with a scalpel or put them together in the same acclimatization pot (Fig. 7a).
3. Carefully transplant the plants in soil pots (see Note 14), inside a greenhouse, without damaging the roots. Water them immediately (Fig. 7b).
4. Cover the box with a clear plastic cover for 30 days to maintain a high air humidity; ensure that the soil always remains moist (Fig. 7c). Potted plants stay over a vermiculite bed (Fig. 7d).
5. Do not expose plants to direct sunlight during the acclimatization step.
6. After 1 month, plants should be grown enough to be transplanted in larger pots and transfer to standard greenhouse conditions (Fig. 7e).

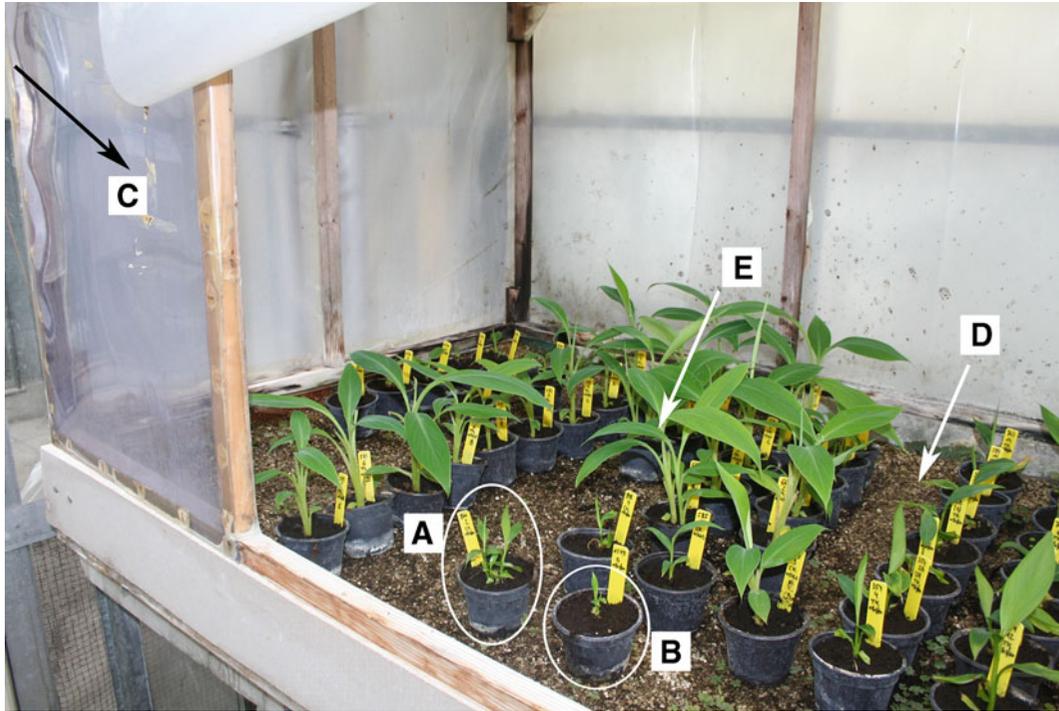


Fig. 7. Greenhouse acclimatization. (a) three plantlets acclimatized together; (b) plantlet size required for acclimatization; (c) plastic box with reclosable plastic door; (d) vermiculite; (e) plant size required to be transferred in standard greenhouse growing conditions.

#### 4. Notes

1. MS medium could be obtained from suppliers as premixed powdered mixtures. This saves a great deal of time and efforts. However, MS medium could also be prepared by dissolving appropriate quantities of macro-, micro-components, and vitamins in distilled water. This protocol is explained in detail for *Musa* by Vuylsteke (15).
2. To reduce the risk of contamination, it is recommended to excise meristems in a laminar air-flow cabinet.
3. No need to work in aseptic conditions at this stage.
4. Care should be taken to avoid cutting the meristem during this procedure.
5. Some aspects of the excision process are critical; among them the maintenance of sterile conditions, the prevention of virus transmission through the excision tools, and the removal of the apical dome without damaging it, in order to ensure its survival.

6. Size of explants is an important factor for the success of the process. Very small explants consisting of only the meristematic dome increase the production of virus-free plants, but they show high mortality rates and grow very slowly.
7. It should be noted that once the dissection process is finished, the meristem begins to lose water rapidly. Therefore, the final steps in the excision process must be completed rapidly in order to avoid excessive dehydration.
8. Dark conditions are required to avoid excessive blackening of tissues caused by photo-oxidation of phenolic compounds. If dark conditions cannot be applied in the room, cover the glass tubes containing the meristems with an aluminum foil.
9. Environmental control is preferred. However, as bananas and plantains can be cultured under a relatively wide range of environmental conditions, complete control is not absolutely necessary. An air-conditioning unit is suitable for temperature control. It is important that the temperature does not fall below 20°C and does not exceed 35°C (15).
10. One or two weeks after excision, the meristems should be inspected under the binocular microscope. Discard contaminated and dead ones. Dead meristems can be recognized by their black appearance and lack of new growth. If contamination exceeds 5%, the disinfection and excision process should be reviewed. Success in meristem-tip culture depends on a large number of factors and is hard to predict. Normally, 2–5% meristems can die as a result of dissection damage and/or a too small size of explants, depending on the skill of the handling person
11. The regeneration process takes at least 3 months or longer according to the plant genotype, meristem size, and culture conditions.
12. Regular subculturing on fresh medium prevents nutrient deficiency, medium dehydration, pH modifications, and accumulation of secondary metabolites which can slow down the growth of meristems.
13. Meristems do not grow fast when transferred onto chemotherapy media and the meristem size remains stable during the 3-month treatment.
14. A sterile soil is not recommended for successful plant establishment. However, a contaminant-free soil should be used.

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## ***Agrobacterium*-Mediated Genetic Transformation of Pineapple (*Ananas comosus* L., Merr.)**

Minal Mhatre

### **Abstract**

Pineapple (*Ananas comosus* L., Merr.) is a commercially important crop, grown in the tropical and subtropical regions. However, the crop is faced with postharvest damage and poor varietal and nutritional improvement. Being a vegetatively propagated crop, conventional breeding programs take longer time for genetic improvement, which may not necessarily successfully develop an improved cultivar. Hence, the genetic modification of pineapple is an alternative handy approach to improve pineapple. We have established an *Agrobacterium*-mediated transformation system using leaf bases from *in vitro*-grown pineapple plants. Being a monocot, acetosyringone is added to the culture medium for overnight growth of *Agrobacterium* and transformation to transfer a gene of interest *MSI99* soybean *ferritin*. Leaf bases isolated from *in vitro* shoot cultures are treated with *Agrobacterium* suspension at two dilutions, 10× and 20×, for 30 min. Explants are subsequently blot dried and cultured on gelrite solidified hormone-free Pin1 medium for 2 days (cocultivation). Periodic transfer is first done to the regeneration medium (Pin1) containing cefotaxime for the suppression of *Agrobacterium* growth. The transformants are selected by culturing on Pin1 medium containing cefotaxime and kanamycin. Multiple shoots, regenerated in leaf bases, are further multiplied and individually rooted in the liquid RM medium amended with antibiotics to recover plants. Putative transformants are analyzed for transgene integration and expression using standard molecular biological methods of PCR, RT-PCR, and genomic Southern.

**Key words:** *Agrobacterium*-mediated transformation of leaf bases, *MSI99* and soybean *ferritin* gene, Molecular analysis of transformants, Pineapple

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### **1. Introduction**

Pineapple, *Ananas comosus* L. Merr., belonging to the family Bromeliaceae, is an important and widely grown fruit crop of high commercial value in the tropical and subtropical areas of the world (1). This is the only self-incompatible species known in the genus and produces fruits containing very hard seeds as high as 3,000. However, by prevention of compatible cross-pollination, seedless parthenocarpic pineapple fruits are produced. Natural crossing

between clones produces seeded fruits. Vegetative propagation by leafy crowns of the fruit or slips or suckers from the parent plant produces clonal plants and that maintain the genetic fidelity of the genotype. Cross-pollination will produce only seedless parthenocarpic fruit.

Pineapple exhibits a high level of genomic heterozygosity and therefore conventional breeding has been difficult to develop improved varieties (2). This makes pineapple breeding a slow process. An  $F_1$  plant takes 4 years to produce fruit. To develop improved varieties and interspecific cross, four backcross generations are necessary to produce a plant suitable for commercial use. Thus, the development of a new cultivar takes 25 years after the initial cross and it then has to be multiplied and distributed (3). Vegetatively propagated pineapple plants produce on an average 4–5 propagules per year and takes a considerable time to produce enough planting material for one hectare starting with a single plant. Alternately, *in vitro* micropropagation of dormant axillary buds from pineapple crowns produces large number of plantlets rapidly and variants obtained *in vitro* are a viable option for cultivar improvement (4). Multiple shoots and plants in *in vitro*-produced leaf bases of pineapple have been successfully achieved (5) and preparation of synthetic seeds is another option for pineapple conservation and micropropagation (6).

Unlike other fruits, pineapple fruit has no starch reserves of its own and sugar accumulates before it is harvested. Therefore, ripened pineapples are picked and get readily spoiled due to fungal infections during handling, storage, and transport. Fusariosis, produced by *Fusarium subglutinans*, develops a serious pineapple disease (7). The pathogen is able to infect all parts of pineapple plant and develops in a characteristic gum (8). Success in the use of preservatives and cold storage to keep pineapples from infection, is limited. Antimicrobial peptides are widely used in nature by various organisms as a component of their host defense mechanism. One such class of antimicrobial peptides, “megainins,” isolated from the skin secretions of African clawed frog *Xenopus laevis*, has antifungal properties, low vertebral and mammalian toxicity. A substitution derivative of megainin, *MSI99*, is reported to inhibit growth and germination of the causal organism of *Fusarium* wilt.

Although plant food contains essential minerals and organic nutrients essential for human nutrition, they are not often present in sufficient amounts. Mostly fruits are consumed fresh and are an excellent source of enriched nutrients. Pineapple, although already a good source of protein, sugar, fiber, and vitamins, can be further fortified with essential nutrients. An iron-storage protein, ferritin, is present in animals, plants, and bacteria and stores up to 4,500 iron atoms.

In view of all this, genetic modification is a viable option to pineapple improvement. We have incorporated successfully an

analogue of the antimicrobial magainin gene *MSI99* to combat postharvest fungal infection (9). The *Agrobacterium* strain EHA 105 harboring plasmids MSI 168 or MSI 164 is employed in the transformation of leaf bases of *in vitro*-derived pineapple shoots. The fortification of pineapple with iron and zinc is done by the incorporation of the soybean *ferritin* gene (10). Our results demonstrate 2.8–5.03 fold increase in iron and 1.52–2.39 fold in zinc among transformed lines in comparison to the controls.

This chapter describes a detailed protocol for efficient *Agrobacterium*-mediated transformation of pineapple leaf bases with *MSI99* and soybean “*ferritin*” gene, as well as molecular analysis of putative transformants with polymerase chain reaction (PCR), RT-PCR, and the genomic Southern of positive lines.

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## 2. Materials

### 2.1. Plant Material, Instruments, Solutions, Culture Media, and Growth Adjuvants

#### 2.1.1. Plant Material

1. Axillary buds, one at the base of each leaf in crowns, of pineapple cv. Queen (see Note 1).
2. Use isolated leaf bases from *in vitro* shoot cultures by peeling them and using only 5–6 mm of the basal portion of the leaf blade for *Agrobacterium* infection.

#### 2.1.2. Instruments and Solutions

1. Mercuric chloride solution (0.1%).
2. Tap water.
3. Standard liquid detergent.
4. Sterile tap water.
5. Sterile Petri plates (glass or plastic), with/without filter paper.
6. Laboratory facilities: Forceps, scalpels with sterile disposable blades, laminar air flow cabinet, wide mouth alcohol bottle and flame, muslin cloth.

#### 2.1.3. Culture Media and Growth Adjuvants

1. Murashige and Skoog (11) basal medium for plant growth, and White’s (12) basal medium for rooting transformed shoots. Media used in *in vitro* work are listed in Table 1 (see Note 2).
2. Antibiotics: cefotaxime (cef) and kanamycin (kan).
3. Acetosyringone (4-acetyl-2,6-dimethoxyphenol, MW 196.2).
4. *Agrobacterium* plasmid vectors: *Agrobacterium* strain EHA 105 harboring plasmids MSI 168 or MSI 164, pSF and pEF-ESF; plasmids MSI 168 or 164 had *ubq 3* gene promoter of *Arabidopsis* and *nos* terminator (13). *Agrobacterium* strain EHA 105 contained the soybean “*ferritin*” cDNA (SF) and

**Table 1**  
**Plant tissue culture media used in *in vitro* experiments**

Medium	Composition <sup>a</sup>
HFMS	MS major and minor salts + MS vitamins + FeEDTA + sucrose 2% (hormone free)
Pin 1	MS major and minor salts + MS vitamins + FeEDTA + sucrose 2% + 1.8 mg/L NAA + 2 mg/L IBA + 2 mg/L Kn
RM	White's major and minor salts + MS vitamins + FeEDTA + sucrose 1% + 0.1 mg/L NAA + 0.4 mg/L IBA

NAA 1-naphthalene acetic acid; IBA indole-3-butyric acid; Kn kinetin; FeEDTA ethylenediaminetetra acetate—ferric salt

<sup>a</sup>Phytigel (2%) was used in HFMS and Pin1 media. RM is liquid medium

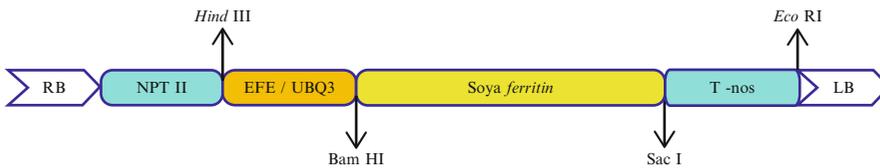


Fig. 1. T-DNA region of a pSF/pEFE-SF. RB and LB are right and left border repeats, respectively; NPT-II is neomycin phosphotransferase; UBQ3 is ubiquitin promoter from *Arabidopsis*; EFE is ethylene-forming enzyme promoter of banana; and T-nos is nos terminator; restriction enzyme sites.

NPT II marker gene (9) driven by *ubq 3* gene promoter of *Arabidopsis* in the plant expression vector pSF. Vector pEFESF has banana “ethylene forming enzyme” (EFE) promoter (Fig. 1).

## 2.2. Plant Cell Transformation

1. Plant material (explants-leaf bases from *in vitro* shoot cultures).
2. *Agrobacterium* strain harboring the desirable gene grown on YENB agar plates.
3. Overnight-grown *Agrobacterium* culture in liquid YENB broth.
4. Hormone-Free Liquid MS Medium (HFMS) for use during leaf base excision and for preparation of *Agrobacterium* suspension.
5. Sterile autoclaved tissue paper sheets.
6. 50 mL conical flasks.
7. Forceps and blades.
8. Sterile Petri plates.
9. Sterile 50 mL centrifuge tubes.

10. Micropipettes and microtips.
11. Parafilm strips.
12. Sterile media in Petri plates: Pin1, Pin1 + cef, Pin1 + cef + kan.
13. Sterile 50% glycerol.
14. Table top centrifuge with rotor to hold 50 mL tubes.

### **2.3. DNA Isolation from Transformed Tissue**

#### *2.3.1. Cetyltrimethylammonium Bromide Method*

1. Sterile mortar-pestle.
2. Forceps and blade holders with blades.
3. Sterile Petri plates.
4. Eppendorf tubes.
5. Micropipettes and microtips.
6. Sterile gloves.
7. Laminar flow unit.
8. Cetyltrimethylammonium Bromide (CTAB) buffer: 50 mM Tris-HCl pH 8.0, 0.7 M NaCl pH 8.0, 20 mM EDTA, CTAB 2%, mercaptoethanol (1  $\mu$ L mL/L).
9. 5 $\times$  TBE buffer (working solution, 1 $\times$ ): Tris-HCl pH 8.0 54 g/L, boric acid 27.5 g/L, EDTA 0.5 M, pH 8.0 20 mL/L.
10. TE buffer (10 $\times$ ): 100 mM Tris, 10 mM EDTA, pH 8.0 (with HCl).
11. Chloroform.
12. Equilibrated phenol.
13. Ethanol 70%.
14. RNase solution.
15. Sodium acetate 3M.

#### *2.3.2. Minipreparation*

1. Microcentrifuge, laminar flow.
2. Sterile gloves.
3. Micropipettes and microtips.
4. Sterile Eppendorf tubes.
5. SYBR green DNA stain.
6. Gel Doc.

### **2.4. Polymerase Chain Reaction**

1. For the PCR mix: sterile Milli-Q water, PCR buffer mix, dNTPs, forward primer, reverse primer, *Taq* DNA polymerase, MgCl<sub>2</sub> solution, DNA template.
2. 500  $\mu$ L PCR tubes.
3. Crushed ice, ice bucket.

4. Micropipettes and microtips, gloves.
5. Thermalcycler: Mastercycler, Eppendorf, USA.

### **2.5. RT-PCR of Positive Lines**

1. RNeasy plant mini kit (Qiagen, USA).
2. cMaster RT-PCR system (Eppendorf, USA).
3. All other requirements of tubes, stain, etc., are as described in previous sections.

### **2.6. Southern Blot Analysis**

1. NC: Hybond N<sup>+</sup> paper, Amersham-Pharmacia, Biotech, UK.
2. pSAN 168 (containing the *MSI99* gene and *nos* terminator).
3. ∞-[<sup>32</sup>P]dCTP Random Primer kit (BRIT, India).
4. Klenow fragment, 500 bp *EcoRI* fragment.
5. Sephadex column, pasteur pipettes, micropipettes, and microtips.
6. Buffers: TE (100 mM Tris, 10 mM EDTA, pH 8.0 with HCl), Na<sub>2</sub>HPO<sub>4</sub> 20 mM, 5% and 7% SDS, saline-sodium citrate (SSC) 20×.
7. Solutions: HCl 0.25 N, NaOH 0.4 M.
8. Dyes: Orange G and Dextran Blue, Tap water.
9. X-ray film, cassette to hold X-ray film + NC, X-ray film developer, cling film.
10. Hood for radioactive work and bin for radioactive waste, protective eye glasses, gloves.

### **2.7. DIG Labeling**

1. *HindIII* restriction enzyme.
2. High Pure PCR Product Purification Kit (Roche Applied Science, Germany).
3. NC: Hybond N+ paper (Amersham-Pharmacia Biotech, UK).
4. DIG High Pure DNA labeling Starter Kit II (Roche Applied Science, Germany).
5. TAE buffer (50×): Tris (242 g), glacial acetic acid (57.1 mL), EDTA (0.5M, pH 8.0, 100 mL); adjust pH to 8.0 and make up volume.

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## **3. Methods**

### **3.1. Plant Media**

#### **3.1.1. Hormone-Free Liquid MS Medium**

1. Prepare MS (11) with all the components in proportion without any growth regulator.
2. Add 2% sucrose and dissolve.

3. Make up the volume with distilled water after pH adjustment to 5.8 and autoclave.

### 3.1.2. Pin1 Medium

1. Prepare MS (11) with all the components in proportion.
2. Add 1.8 mg/L 1-naphthalene acetic acid (NAA), 2 mg/L indole-3-butyric acid (IBA), 2 mg/L kinetin (Kn) to the MS mix.
3. Add 2% sucrose and dissolve.
4. Adjust the pH to 5.8 and make up the volume with distilled water.
5. Add phytigel (0.2%) and autoclave.

### 3.1.3. RM Medium

1. Prepare White's (12) basal medium containing 1% sucrose, 0.1 mg/L NAA, 0.4 mg/L IBA, without phytigel.
2. Adjust pH to 5.8 and dispense into tubes. The medium in each tube is layered with filter paper strips (folded in such a way as to support the shoot for rooting) and autoclaved (4).

### 3.1.4. YENB Medium

This medium is to be used for plating and for the overnight growth of *Agrobacterium*. It contains 0.75% yeast extract and 0.8% nutrient broth in distilled water. To prepare overnight-grown *Agrobacterium* culture:

1. Add 50 mg/L sterile kanamycin and 10  $\mu$ L acetosyringone to 10 mL autoclaved liquid YENB medium.
2. To prepare solidified YENB medium for growing *Agrobacterium* colonies in Petri plates, add phytigel (0.2 g/100 mL) to liquid YENB, dissolve, and autoclave.
3. When medium is lukewarm, add sterile kanamycin (50 mg/L) and pour into Petri plates (see Note 3).

## 3.2. Preparation of Media Components

### 3.2.1. Antibiotics

1. Prepare stocks of cefotaxime (1 g/10 mL) and kanamycin (5 g/50 mL) by dissolving in distilled water.
2. Filter sterilize each and aliquot and store in freezer.
3. Use 400 mg/L cefotaxime and 50 mg/L kanamycin in YENB or Pin1 medium.
4. In RM medium, add 400 mg/L cefotaxime and 100 mg/L kanamycin.

### 3.2.2. Acetosyringone: (4-acetyl-2,6-dimethoxy-phenol, MW 196.2)

1. Prepare this solution in ethyl alcohol, stock 20 mg/L (final concentration 100  $\mu$ m). Use 10  $\mu$ L/10 mL.

### 3.2.3. *Agrobacterium* Plasmid Vectors

The *Agrobacterium* strain EHA 105 harboring plasmids MSI 168 or MSI 164, pSF and pEFESF is used to transform leaf bases of *in vitro*-derived pineapple shoots. *Agrobacterium* strain EHA 105

harboring plasmids MSI 164 or 165 has *ubq 3* gene promoter of *Arabidopsis* and *nos* terminator (13). Another construct of *Agrobacterium* strain EHA 105 contain soybean “*ferritin*” cDNA (SF) and NPT II marker gene (9) driven by *ubq 3* gene promoter of *Arabidopsis* in the plant expression vector pSF. Construct vector pEFESF by cloning the SF under banana EFE promoter (Fig. 1).

#### 3.2.4. Surface Sterilization of Dormant Buds from Pineapple Crowns

1. After surface sterilization of pineapple crowns, scoop out the dormant axillary buds and tie them in a muslin cloth.
2. Wash with soap and tap water twice.
3. In the laminar flow, soak in mercuric chloride (0.1% soln.) for 3 min, rinse with sterile tap water three or four times.
4. Decant off the tap water, and blot dry the buds in Petri plates on sterile filter paper.

### 3.3. Culture Conditions for In Vitro Plant Tissues

1. All media are solidified with 0.2% phytigel (Sigma, Cat # P-8169) except liquid media wherever specified.
2. The pH of the medium is adjusted to 5.8 using 0.1N HCl or 0.1N NaOH.
3. All media are autoclaved at 121 °C, 15 lbs pressure for 20 min.
4. *Agrobacterium tumefaciens* cultures are grown overnight in incubator-shaker (90 rpm) at 27 °C.
5. All plant cultures are maintained at 25 ± 2 °C in 16 h photoperiod with light (12.1 μmol/m<sup>2</sup>/s irradiance at culture level) provided by Philips fluorescent tubular lamps (Philips slim lite, 36W, India) and 60–70% relative humidity.

### 3.4. Preparation of Stock Shoot Cultures

1. In vitro cultures are used for shoot multiplication by placing nodal portions (0.5 cm), previously established from dormant axillary buds of plants in the field, on Pin1 medium. The nodal portions do not require surface sterilization and are cultured as such after excision. Basal ends of nodal portions are embedded vertically in the medium.
2. Leaf bases from such established shoots are used as explants for transformation.

### 3.5. Plant Cell Transformation

#### 3.5.1. Streaking of *Agrobacterium* Cultures on YENB

1. *Agrobacterium* constructs pSF, pEFESF, and MSI 168/164 are streaked on YENB plates, sealed with parafilm and incubated at 27 °C for 2 days.
2. After 2 days, the growth of colonies is seen (see Note 4).

#### 3.5.2. Preparation of Overnight-Grown *Agrobacterium* Cultures

1. One loopful of the *Agrobacterium* colonies, growing in Petri plates, is cultured in 10 mL liquid YENB medium containing kanamycin (50 mg/L) as a selective marker.

2. Acetosyringone (10  $\mu$ L) is added to this medium for the activation of the *vir* genes that dictate attachment to the plant cell.
3. After *Agrobacterium* inoculation, culture is incubated overnight in an incubator-shaker at 27°C, 150 rpm.

#### 3.5.3. Preparation of Explants for Transformation

1. Leaves from *in vitro* raised pineapple stock cultures are peeled off one by one. The basal portion of the leaf blade, which appears to be translucent after peeling, is cut and floated on MS liquid medium to be used as explant for transformation.
2. Two dilutions of overnight-grown *Agrobacterium* culture (10 $\times$  and 20 $\times$ ) are used to transform the leaf bases with *ferritin* and *MSI99* in separate experiments (see Note 5).

#### 3.5.4. Transformation of Leaf Bases

1. Isolate leaves by carefully peeling them from *in vitro* shoots. Excise 5–6 mm of the translucent basal portion of each leaf blade and float onto liquid HFMS medium in a sterile Petri plate.
2. Centrifuge the overnight-grown *Agrobacterium* culture in 50 mL centrifuge tube at 11,000 rpm for 15 min (see Note 6).
3. Discard the supernatant and add 10 mL liquid HFMS medium to the pellet.
4. Mix the pellet by gentle shaking.
5. Take 1 mL of this suspension in a sterile 50 mL conical flask and add 9 mL MS liquid medium to get 10 $\times$  dilution. To 0.5 mL of the same suspension in another 50 mL sterile conical flask, add 9.5 mL liquid HFMS medium to get 20 $\times$  dilution. Add 10  $\mu$ L acetosyringone to each.
6. Pipette out the MS liquid medium from the Petri plates containing leaf bases and divide the explants into two separate dishes. Add the diluted (10 $\times$  and 20 $\times$  dilutions) *Agrobacterium* mixtures (one each) to the Petri plates.
7. Allow to stand for 30 min to enable transfer of DNA into the cells of the leaf bases.
8. After 30 min, pipette off the HFMS liquid medium, pick up leaf bases from both the dilutions one by one and place on separate sterile autoclaved filter papers for each of the dilutions. This is to pat dry the leaf bases to remove excess *Agrobacterium* suspension. Cover it with several layers of sterile autoclaved filter papers. Leave them to dry for 40 min. Inoculate pat dried leaf bases (50–60) on antibiotic-free Pin1 medium in Petri plates for cocultivation. Seal with parafilm and leave for 2 days (see Note 7; Fig. 2a).



Fig. 2. *Agrobacterium*-mediated transformation of pineapple leaf bases. (a) Leaf base explants transformed with MS1168; (b) regenerating explants on Pin1 + cef + kan medium; (c) multiplication of transformed shoots on Pin1 + cef + kan medium; (d) rooting of transformed shoot on RM medium; (e, f) transformed plants in cups and pots; (g, h) fruiting plant and harvested fruits of transformed (*MS199*) plants.

9. On the third day, transfer the leaf bases onto Pin1 + cefotaxime medium for suppression of *Agrobacterium* growth. Seal and keep for 3 days.
10. After 3 days, transfer the leaf bases to Pin1 + cefotaxime + kanamycin medium for selection of putatively transformed explants.
11. Subsequently the explants are subcultured every week onto the fresh medium of the same composition and allowed to grow until multiple shoots are regenerated in the leaf bases (see Note 8; Fig. 2b).
12. Transfer multiple shoots, 2 cm long, regenerated from the explants onto Pin1 medium containing cefotaxime and

kanamycin in test tubes. This is done to multiply the shoots to obtain enough material for DNA isolation and analysis of putative transformants (Fig. 2c).

13. Transfer shoots 4–5 cm long in liquid RM medium containing cefotaxime and kanamycin (100 mg/L) for root induction. In 6 weeks exuberant root growth is seen (see Note 9; Fig. 2d).
14. Transfer rooted plants 5–6 cm long first in paper cups filled with Soilrite (Soilrite mix, TC; mixture of 75% Irish peatmoss and 25% expanded Perlite, M/s. Chougule Industries Ltd., Mumbai, India) and later in pots filled with soil. After field transfer, plants start producing normal fruits within 12–14 months (Fig. 2e–h).

### **3.6. DNA Isolation from Transformed Tissue**

There are several standard methods for plant DNA isolation. Generally the CTAB method is widely used as well as miniprep method (14).

#### *3.6.1. Cetyltrimethylammonium Bromide Method*

1. Take 3 g leaf from transformed pineapple plant.
2. Grind tissue to powder in liquid nitrogen. Add CTAB 2× buffer (2 mL) per gram of tissue.
3. Incubate mixture at 65 °C for 1 h with occasional inversion.
4. Add equal volume of chloroform to the above mixture and separate the two phases by centrifuging at 15,000 rpm, 10 min at 4 °C.
5. Transfer the upper aqueous phase to a fresh tube. To this, add equal volume of isopropanol and mix gently by inversion.
6. Pellet the genomic DNA by centrifugation at 15,000 rpm for 10 min at 4 °C; vacuum dry the pellet for 5 min and dissolve in few µL of TE buffer.
7. Remove RNA by adding RNase to a final concentration of 10 µg m/L and incubate at 37 °C for 30 min.
8. Add equal volume of equilibrated phenol, mix and centrifuge at 10,000 rpm for 5 min at 4 °C.
9. Collect upper aqueous layer in a fresh Eppendorf tube and to this add equal volume of phenol chloroform (1:1) mixture, centrifuge 10,000 rpm, 5 min at 4 °C.
10. Add equal volume of chloroform to the aqueous phase and centrifuge at 12,000 rpm for 5 min.
11. Add to the upper aqueous phase 1/10 vol. of sodium acetate and 2 vol. of ethanol and centrifuge at 15,000 rpm 15 min.
12. Wash the pellet once with 1 mL 70% ethanol.
13. Centrifuge at 15,000 rpm for 12 min, vacuum dry pellet for 5 min and dissolve in 50 µL of TE buffer.
14. Store at –20 °C.

### 3.6.2. Minipreparation

Gen Elute Plant Genomic DNA minipreparation kit (Sigma cat. # G2N 350) is used to isolate DNA from the putative pineapple transformants. As per manufacturer's instructions, putative transformants are analyzed as: MSI168/164; EFESF; ubqSF; controls (-ve, untransformed tissue; +ve, plasmid). The kit is supplied with following: Lysis A solution 450  $\mu$ L; lysis B solution 50  $\mu$ L; precipitation solution 125  $\mu$ L; binding solution 700  $\mu$ L; washing buffer 500  $\mu$ L; elution buffer 100  $\mu$ L; tubes and columns.

1. Weigh  $\approx$ 200 mg leaf tissue and grind it using liquid nitrogen to a fine powder in a sterilized mortar-pestle.
2. To the fine powder, add 450  $\mu$ L of lysis A solution, mix well and transfer to a sterile Eppendorf tube.
3. To this, add 50  $\mu$ L of lysis B solution, mix well. White precipitate of carbohydrates, lipids, proteins (except nucleic acids which will be in the aqueous phase) will be observed.
4. Remove RNA by adding RNase to a final concentration of 10  $\mu$ g m/L and incubate at 37 °C for 30 min.
5. Keep this at 65 °C for 10 min. Add 125  $\mu$ L of precipitation solution and mix it well.
6. Keep the tubes on ice for 5–10 min.
7. Centrifuge at 10,000 rpm for 1 min.
8. Transfer the supernatant in a blue-colored filtration column tube. Centrifuge at 10,000 rpm for 1 min.
9. Discard the filter and take the flow-through liquid into a fresh autoclaved Eppendorf tube. Add 700  $\mu$ L of the binding solution and mix well.
10. From this, transfer 700  $\mu$ L into a red ringed column fitted in a 2 mL-collection tube. Centrifuge at 10,000 rpm for 1 min.
11. Discard the flow-through liquid and add the remaining solution to the same column. Centrifuge it at 10,000 rpm for 1 min.
12. Discard the flow-through liquid along with the collection tube and transfer the column to a new 2 mL-collection tube.
13. Add 500  $\mu$ L of washing buffer (diluted with ethanol) to the column and centrifuge 10,000 rpm, 1 min. Repeat this again.
14. Carry out blank centrifugation for 10,000 rpm for 2 min (to remove the alcohol). After centrifugation, retain the column and fix the column to a new 2 mL-collection tube (remove the cap of the tube).
15. Add 100  $\mu$ L of elution buffer (pre-heated at 65 °C) to the column. Centrifuge at 10,000 rpm for 1 min. This elute is DNA. Discard the column, transfer the DNA in a fresh tube and store it at -20 °C.
16. Check the isolated DNA on 0.8% agarose gel by electrophoresis (use SYBR green DNA stain).

### 3.7. Polymerase Chain Reaction

For analyzing tissues transformed with *MSI99*, the upstream primer used for PCR is specific to the secretory signal sequence (P168), and the 3'-end primer is specific to the 5' region of the *nos* terminator (*Pnos*). The primer sequences used are same as described previously (13). With the *ferritin* constructs, using “ferritin” and NPT II-specific primers, confirmation of transformation is carried out by PCR. Primer sequences are given below:

1. 5'GGATCCAACAATGGCTCTTGCTCCATCCAAAGTT3'.
  2. 5'GAGCTCGGCTATTCAAGATTAAGCAGCATC3'.
1. Fill out a PCR worksheet with the sample identifiers of all samples to be used. Following the master mix template sheet (Table 2), calculate the amount of each component needed for the total number of samples + 2 (see Note 10).
  2. Label 200  $\mu$ L tubes/strip tubes with sample identifier numbers from the PCR worksheet, so that the numbers can be cross-referenced with identifiers and each PCR reaction tube can be individually identified. PCR tubes can be placed in a freezer rack to keep cold (see Note 11).
  3. Prepare the Master mix (MM) in a 1.5 mL tube, adding each component in the order listed on the PCR form (Table 2): water, 10 $\times$  buffer, dNTPs, MgCl<sub>2</sub>, then primers. Shake contents down into the bottom of the tube before pipetting them into the MM (see Note 12).

**Table 2**  
**Reaction mixture (Master mix, MM)<sup>b</sup> used in PCR reactions**

Reaction mixture ingredients	For 1 tube ( $\mu$ L)
Sterile distilled water	29
PCR Buffer mix	5
dNTPs	2.5
MgCl <sub>2</sub>	1.5
Forward primer	1.0
Reverse primer	1.0
Taq DNA polymerase	0.5
Volume	40
DNA template (add to each sample)	10
Total volume	50

<sup>b</sup>MM does not contain the DNA template. Template DNA is added to individual samples after aliquoting the MM

4. Add the correct amount of *Taq* DNA polymerase to the MM and close the tube. Pipette out the MM into your labeled 200  $\mu$ L-PCR tubes/strip tubes (usually 40  $\mu$ L per tube) with the pre-PCR 200- $\mu$ L pipette. Mix the MM solution well (see Note 13).
5. Add 10  $\mu$ L of each template DNA in order. Close caps of all tubes firmly and place the tubes into the thermal cycler (see Note 14).
6. Start the program (5 min at 94 °C, followed by 34 cycles of 1 min at 94 °C, 1.5 min at 58 °C and 1 min at 72 °C. The cycle is concluded with additional 10 min at 72 °C, then hold at 4 °C). Most PCR programs take about 2 h.
7. After the PCR is complete, the amplified product is checked by running it through 0.8% agarose gel using SYBR™ green DNA gel stain (Invitrogen, Cat # S33102) 10  $\mu$ L/100 mL. The gel can be viewed under UV transilluminator and photographed using a Gel Doc. A ferritin-specific band of 780 bp is observed in the transformed plants of both the constructs, while being absent in the untransformed control plants (Fig. 3).

### 3.8. RT-PCR of Positive Lines

1. Randomly select PCR-positive lines and untransformed control lines for reverse transcription (RT)-PCR analysis to determine the transgene expression. Isolate leaf tissue.
2. Extract total RNA from the leaf tissue using RNeasy plant mini kit (Qiagen, USA) and subsequently clean up by DNase treatment (as per manufacturer's instructions).
3. As per manufacturer's instructions use 1–5  $\mu$ g aliquot of total RNA for cDNA synthesis using cMaster RT-PCR system (Eppendorf, USA).

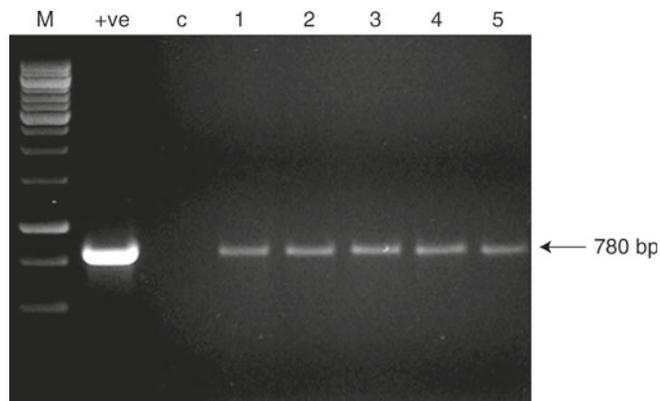


Fig. 3. PCR products of pineapple transformed (*ferritin*) lines. M, marker; +ve, positive control; C, untransformed (–ve control); lanes 1–3, individual transformants of EFESF; lanes 4 and 5, individual transformants of UbqSF.

- Use 10  $\mu\text{L}$  of this cDNA as template for PCR with soybean *ferritin*-specific primers described above. Carry out the following thermal cycling conditions: 94 °C for 5 min and subsequently 30 cycles each comprising 94 °C for 1 min, 58 °C for 1 min, and 72 °C for 2 min with a final extension at 72 °C for 10 min. Determine expression by the amplification of the 780 bp fragment.

### 3.9. PCR/Genomic Southern Blot Analysis

#### 3.9.1. Southern Analysis (*MSI99*)

The PCR products after gel separation are blotted onto nylon membranes (Hybond N+; Amersham-Pharmacia). The 500 bp *EcoRI* fragment from pSAN168 containing the *MSI99* gene and *nos* terminator was radioactively labeled with  $\alpha$ -[ $^{32}\text{P}$ ]dCTP (Table 3) using the Random Primer Kit (BRIT, India) according to the manufacturer's instructions for hybridization. A general procedure for blotting and subsequent hybridization was carried out as described (15), with minor changes as below. PCR-Southern and genomic Southern of transformed (*MSI99*) pineapple plants is illustrated (Figs. 4 and 5).

Southern Hybridization with Labeled Probe

- Run gel as usual. Rinse in 0.25 M HCl until blue dye starts to turn yellow.
- Rinse twice in distilled water.
- Rinse in 0.4 M NaOH till dye regains color.
- Transfer to nylon (N+) membrane overnight in NaOH–NaCl (8 g NaOH + 2 g NaCl in 500 mL distilled water).
- Next day, rinse the membrane in 2 $\times$  SSC buffer (20 $\times$ : 175.3 g sodium chloride + 88.2 g Sodium citrate, in 800 mL distilled water, pH 7.0 with NaOH, make up volume to 1 L) 5 min.
- Cross-link the membrane and store.

**Table 3**  
**Preparation of probe ( $\text{P}^{32}$  labeling)**

	Tube 1. MW marker 2 $\mu\text{L}$	Tube 2. 168 fragment 5 $\mu\text{L}$
Milli-Q water	20 $\mu\text{L}$	17 $\mu\text{L}$ (Boil at 95 °C, 5 min; chill on ice)
Then add individually to both tubes		
R primer buffer	5 $\mu\text{L}$	5 $\mu\text{L}$
R primer	5 $\mu\text{L}$	5 $\mu\text{L}$
dATP, dGTP, dTTP	4 $\mu\text{L}$ each	4 $\mu\text{L}$ each
Finally add these two components (work behind hood)		
Labeled $\text{P}^{32}$ dCTP	4 $\mu\text{L}$	4 $\mu\text{L}$
Enzyme (Klenow fragment)	2 $\mu\text{L}$	2 $\mu\text{L}$

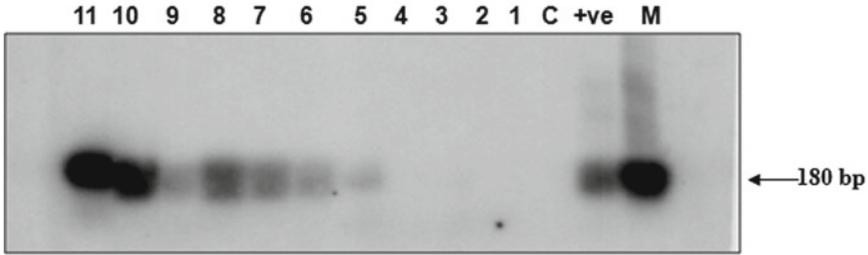


Fig. 4. PCR-Southern of transformed (*MSI99*) pineapple plants. M, marker; +ve (positive control); C, untransformed (–ve control); lanes 1–4, non-transformed plants; lanes 5–11, transformed plants.

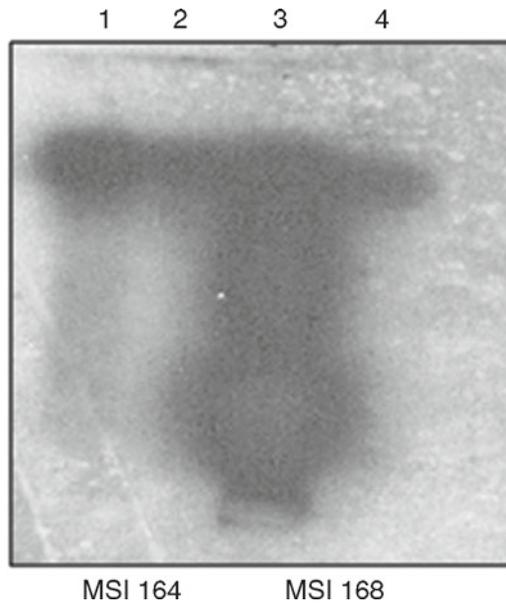


Fig. 5. Genomic Southern of transformed (*MSI99*) plants. Lanes 1 and 3, C+ positive control; lane 2, MSI164 transformed line; lane 4, MSI 168 transformed line.

#### *For prehybridization*

1. Prehybridize the membrane in 0.25M  $\text{Na}_2\text{HPO}_4$  buffer (pH 7.0) containing 7% SDS for 30 min.
2. Prepare and add labeled probe. Hybridize overnight.
3. Keep both samples at 37°C for 1–2 h.
4. Purify by passing through Sephadex column (gel filtration). Work behind hood.
5. Rinse pasteur pipette (without neck constriction) with  $\approx 2$  mL alcohol. Fix column on stand. Wash with 2 mL TE buffer. Collect in beaker. Add 3–4 mL Sephadex 100 (in TE) and fill pipette. Keep collecting.

6. Mix 50  $\mu\text{L}$  dye (Orange G+ Dextran Blue) with radiolabeled samples 1 and 2 (separately in each).
7. Load sample 1 onto column and wash with 1–2 mL TE buffer. Let 1–2 drops pass and then collect in the respective sample tube till all blue color is collected. Stop when drop starts turning orange yellow. Repeat with sample 2 in separate column. Discard the pipettes as radioactive waste.
8. Heat samples 1 and 2 at 95 °C for 5 min. Cover with parafilm and foil.
9. Add to NC (Hybond N<sup>+</sup> paper, Amersham-Pharmacia), keep overnight at 42 °C.
10. Wash in 20 mM Na<sub>2</sub>HPO<sub>4</sub> buffer + 5% SDS twice, 20 min each, and with 20 mM Na<sub>2</sub>HPO<sub>4</sub> buffer + 1% SDS once for 10 min. Check counts. If same, put on shaker at 55 °C, 15–20 min. Cover the nylon membrane with cling film. Expose to X-ray film in cassette. Keep for 3–4 h.
11. Develop film with developer solution (standard method) and rinse with tap water.

#### DIG Labeling

The plants transformed with pSF and pEFESF (soybean *ferritin* gene) are similarly blotted. Labeling is carried out using DIG labeling kit as per manufacturer's instructions. Follow a standard protocol using the above kits and enzymes. Southern hybridization confirms the stable integration of the transgene.

1. Digest genomic DNA (20  $\mu\text{g}$ ) overnight with *Hind*III restriction enzyme.
2. Purify the digested genomic DNA using High Pure PCR Product Purification Kit (Roche Applied Science, Germany) and separate overnight at the field strength of 1.25 V/cm in a 1% (w/v) agarose TAE gel.
3. Transfer DNA to a nylon membrane Hybond N+ (Amersham-Pharmacia Biotech, UK) using standard protocols (16). Immobilize restricted DNA on the membrane by baking at 120 °C for 30 min.
4. Hybridize this DNA against a random primed DIG-labeled probe of 780 bp fragment obtained by PCR amplification of pSF with primers mentioned earlier.
5. Perform chemiluminescent detection of hybridization signals using DIG High Pure DNA labeling Starter Kit II (Roche Applied Science, Germany) according to manufacturer's instructions.
6. The *ferritin* DIG-labeled cDNA shows the hybridization in both pSF- and pEFESF-transformed lines, while being absent in untransformed control plants. The pEFE-SF-transformed lines show the integration of four copies of the transgene. The pSF-transformed lines show the integration of three copies (9).

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## 4. Notes

1. After surface sterilization of pineapple crowns, scoop out the dormant axillary buds and tie them in a muslin cloth. Wash with soap and tap water twice. In the laminar flow, soak in mercuric chloride (0.1%) for 3 min, rinse with sterile tap water three or four times. Decant off the tap water and blot dry the buds on sterile filter paper in Petri plates.
2. Several companies supply ready-to-use mix sachets of basal media, which can be dissolved in distilled water for media preparation according to the instructions of the manufacturer. Alternatively, use 20× stock solutions of major and minor salts, vitamins, and FeEDTA prepared in proportion (11, 12). Use filter-sterilized stock solutions of plant growth regulators (1 mg/L).
3. YENB should be autoclaved without any antibiotics. Antibiotics should be filter-sterilized and added to autoclaved YENB medium.
4. After colonies are observed, few plates can be stored in the refrigerator as stock cultures of *Agrobacterium* strains.
5. This helps to determine the *Agrobacterium* dilution which is the most conducive to transform the explants, since this can vary as per the cultivar used.
6. To 700 µL of overnight-grown *Agrobacterium* culture, add 300 µL autoclaved 50% glycerol in a sterile 1.5 mL Eppendorf tube. Mix well, cover with parafilm, and keep at -20 °C. When required, 100 µL of this can be thawed and plated on YENB + 50 mg/L kanamycin solid medium to grow *Agrobacterium* colonies. Streaking should be periodically done on YENB + 50 mg/L kanamycin medium to maintain viability of the *Agrobacterium* constructs.
7. Place some weight on the tissue papers laid over the explants. This will help to completely blot off the adhering *Agrobacterium* suspension.
8. For cocultivation, the *Agrobacterium*-treated explants are cultured on Pin1 medium without antibiotics. Pin1 medium containing 400 mg/L cefotaxime is used for suppressing the growth of *Agrobacterium* post cocultivation. Pin1 + 400 mg/L cefotaxime + 50 mg/L kanamycin is used for the selection of transformants. After few subcultures, the explants may appear darken. Nevertheless, they should be subcultured. Darkened explants also produce multiple shoots. Experiments should be repeated to achieve high frequency of transformation. All media are plated onto presterilized disposable plastic Petri plates (Laxbro, India), sealed with parafilm and stored.

9. Concentration of kanamycin is increased to 100 mg/L in RM medium to select true transformants and eliminate any escapes.
10. By this, components are provided in excess of the required amount and any pipetting error is nullified. Thus, none of the components of the PCR Master Mix are limiting during amplification.
11. Remember to include a tube at the end for the “negative control” (a tube with no template DNA, added to check for contamination).
12. Be sure to mix the PCR Master Mix well before aliquoting it into the sample tubes.
13. It is not necessary to shake up the *Taq* DNA polymerase before pipetting, but you may want to spin down the *Taq* tube to force contents to the bottom. Pump up and down to rinse *Taq* DNA polymerase into the Master Mix.
14. If liquid is not well settled in the bottom of the tubes, spin down all extract tubes before opening.

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## Protocol for Inducing Flower Color Somaclonal Variation in *Torenia* (*Torenia fournieri* Lind.)

Duong Tan Nhut, Nguyen Thanh Hai, Pham Thi Minh Thu, Nguyen Ngoc Thi, Truong Thi Dieu Hien, Tran Trong Tuan, Nguyen Ba Nam, Nguyen Phuc Huy, Hoang Xuan Chien, and Shri Mohan Jain

### Abstract

White or light purple flower color *Torenia* (*Torenia fournieri* Lind.) varieties were successfully developed from the parental variety having violet flowers. This was accomplished by reducing Fe micronutrient in the culture media for the induction of in vitro flowering. The flower induction was highest in modified Murashige and Skoog (MS) medium containing ½ strength of macroelements, microelements, organic additives, and full Fe (M1) when compared to MS medium containing ½ strength of macronutrients, micronutrients, full Fe, and full organic additives (M2). The flower color was stable in two new *Torenia* varieties through three generations ex vitro. The results showed a wide range of somaclonal variation in flower colors; early flowering occurred in MS medium containing ½ strength of macroelements, microelements, Fe, and full strength of organic additives (M3). The selection of desirable somaclones and their micropropagation in subsequent generations led to the development of new and stable *Torenia* lines.

**Key words:** Fe micronutrient, Flow cytometry, Flower color, Somaclonal variation, Thin cell layers

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### 1. Introduction

Somaclonal variation is quite common in tissue culture-derived plants (1), which is attributed to preexisting genetic variation in somatic cells, single gene mutations, aneuploidy, type of explants, age of the donor plants, type of plant growth hormones, age of culture, genotype, and transposable elements (2, 3). They result from altered gene expression and usually irreversible, and segregation occurs in Mendelian fashion. The alteration in the genetic information would give rise to genetically stable lines. Nongenetic changes appear more frequently, and are reversible and predictable (4).

Therefore, the genetic stability of the selected somaclones should be ascertained before put to use in plant breeding or commercial production. Somaclones showed differences in flower morphology, flower size, flower color, and number of flowers per plant in *Begonia* × *elatior*, *Saintpaulia ionantha* (2, 4–6), and *Phalaenopsis* and *Doritaenopsis* (7).

*Torenia* (*Torenia fournieri*) belongs to family Scrophulariaceae, is one of the most commercially viable bedding plants. *Torenia* is often called Wishbone flower, some species are grown as garden plants; flower color ranges from white with yellow throats to violet, blue, cobalt, lavender, and purple.

Interactions between mineral elements in the culture medium are major factors affecting plant nutrient balance (8). Iron (Fe) is an essential element for normal plant growth and development. It plays a pivotal role in numerous physiological processes and essential cofactor for many metalloproteins. Terry (9) demonstrated that Fe stress influence photosynthesis mainly by altering the capacity of leaves for light harvesting and electron transport (photochemical capacity). Subsequently, Taylor et al. (10) showed effect of Fe nutrition on the activities of three regulatory enzymes of photosynthetic carbon metabolism. In the present study, we investigated the effect of Fe micronutrient on the modification of *Torenia* flower color by using modified MS medium containing ½ strength of macronutrients, micronutrients, Fe, and full organic additives.

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## 2. Materials

### 2.1. Plant

1. *Torenia* (*T. fournieri* Lind.) variety with violet flowers was used as explant source.

### 2.2. Chemicals

1. All chemical and organic ingredients and plant growth regulators (PGRs) (see Table 1).
2. MgCl<sub>2</sub>, DAPI (4, 6-diamidino-2-phenylindole), Triton X-100, Tris-HCl buffer at pH 7.5 (see Table 2).
3. 0.1% HgCl<sub>2</sub>.
4. Ethanol and doubled distilled water.

### 2.3. Culture Media

1. Phytigel (Sigma).
2. Culture media: Modified MS (11) media: (a) ½ strength macroelements, microelements, organic additives, and full strength of Fe (M1), (b) ½ strength macroelements, microelements, full Fe, and organic additives (M2), and (c) ½ strength macroelements, microelements, Fe, and full strength organic additives (M3) (see Table 1).

**Table 1**  
**Medium composition for various activities**

Ingredients	MS	M1	M2	M3
<b>Macronutrients</b>				
Ammonium nitrate ( $\text{NH}_4\text{NO}_3$ )	1,650 mg	825 mg	825 mg	825 mg
Calcium chloride anhydrous ( $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ )	440 mg	220 mg	220 mg	220 mg
Potassium nitrate ( $\text{KNO}_3$ )	1900 mg	950 mg	950 mg	950 mg
Potassium phosphate ( $\text{KH}_2\text{PO}_4$ )	170 mg	85 mg	85 mg	85 mg
Magnesium sulfate ( $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ )	370 mg	185 mg	185 mg	185 mg
<b>Micronutrients</b>				
Boric acid ( $\text{H}_3\text{BO}_3$ )	6.2 mg	3.1 mg	3.1 mg	3.1 mg
Cobalt chloride ( $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ )	0.025 mg	0.0125 mg	0.0125 mg	0.0125 mg
Manganese sulfate ( $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ )	22.3 mg	11.15 mg	11.15 mg	11.15 mg
Cupric sulfate ( $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ )	0.025 mg	0.0125 mg	0.0125 mg	0.0125 mg
Potassium iodide (KI)	0.83 mg	0.415 mg	0.415 mg	0.415 mg
Sodium molybdate ( $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ )	0.25 mg	0.125 mg	0.125 mg	0.125 mg
Zinc sulfate ( $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ )	8.6 mg	4.3 mg	4.3 mg	4.3 mg
<b>Iron</b>				
Ferrous sulfate ( $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ )	27.8 mg	27.8 mg	27.8 mg	13.9 mg
$\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$	37.2 mg	37.2 mg	37.2 mg	18.6 mg
<b>Organic additives</b>				
Myo-inositol	100 mg	50 mg	100 mg	100 mg
Niacin	0.5 mg	0.25 mg	0.5 mg	0.5 mg
Pyridoxine · HCl	0.5 mg	0.25 mg	0.5 mg	0.5 mg
Thiamine · HCl	0.1 mg	0.05 mg	0.1 mg	0.1 mg
NAA (alpha-Naphthaleneacetic acid)	0.1 mg	0.1 mg	0.1 mg	0.1 mg
BA (6-Benzylaminopurine)	0.2 mg	0.2 mg	0.2 mg	0.2 mg
Sucrose	30 g	30 g	30 g	30 g
Phytigel	2.5 g	2.5 g	2.5 g	2.5 g

**Table 2**  
**Ingredients of DAPI stain solution**

Tris-HCl buffer at pH 7.5	10 mM
Triton X-100	0.1%
$\text{MgCl}_2$	2 mM
DAPI (4,6-diamidino-2-phenylindole)	2 mg/L

**2.4. Flow Cytometry**

1. Flow cytometer CA II (Partec Ltd., Munster, Germany).

**2.5. Tissue Culture Instruments**

1. Scalpel.
2. Forceps.
3. Glassware.
4. Plastic ware.
5. pH meter.
6. Autoclave.

**2.6. Greenhouse Facilities**

1. Humidity control.
2. Soil: peat mixture.
3. Pots for growing plants.
4. Light control.
5. Tap water.

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**3. Methods**

**3.1. Setting Up Medium and Conditions**

1. Preparation of Fe-EDTA solution: Dissolve 98.1 g EDTA in 1 N NaOH and 83.4 g FeSO<sub>4</sub>·7H<sub>2</sub>O in 1 L distilled water. Add EDTA solution to Fe solution and aerate until turns dark brown in a dark-colored bottle. Dissolve completely 1 mL Fe solution into ½ strength MS medium and make up to 1 L medium M3.
2. Adjust pH of media to 5.8 with 1 M KOH before autoclaving at 121°C, 1 atm for 20 min. Add 2.5 g/L Phytigel (Sigma) for solidifying the medium unless otherwise stated.
3. Incubate cultures in growth chambers, adjusted to 25 ± 2°C and 75–80% relative humidity, photosynthetic photon density flux (PPDF) 45 mmol/m<sup>2</sup>/s with a photoperiod of 10 h per day.

**3.2. Sterilization**

1. Wash leaves and stems of *Torenia* thoroughly under running tap water for 30 min, soak in detergent for 10 min, and rinse six times with distilled water.
2. Treat segments with 70% ethanol for 1 min.
3. Wash with sterile distilled water and disinfect with 0.1% HgCl<sub>2</sub> aqueous solution for 6 min and then rinse six times in sterile distilled water.

**3.3. Thin Cell Layer Cutting**

1. The *Torenia* leaves and stems are cut into transverse thin cell layers (tTCL) by serial free hand sections (approximately 0.5 mm thick) by a scalpel blade under aseptic conditions.
2. The shoots are cultured in 100 mm × 20 mm Petri dishes containing 40 mL semi-solid medium.

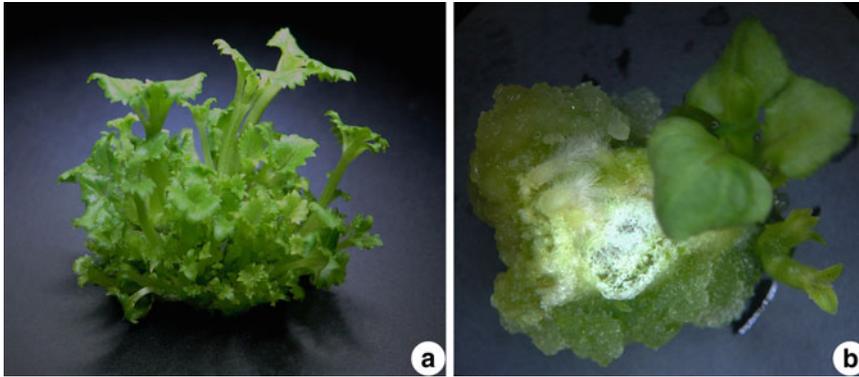


Fig. 1. Adventitious shoots regenerated from leaf (a) and stem (b) of *Torenia fournieri* Lind.

### 3.4. Culture Procedure

#### 3.4.1. Shoot Formation In Vitro

1. Place tTCLs of the leaf and stem in Petri dishes ( $\varnothing$  10 cm) containing MS medium supplemented with 0.2 mg/L Benzylaminopurine (BAP), 0.1 mg/L Naphthalene- $\alpha$ -acetic acid (NAA), 30 g/L sucrose, and 2.5 g/L phytigel. Shoots initiate around the epidermal cells of TCLs after 3 weeks of culture without callus phase (see Fig. 1).
2. After 8 weeks of culture initiation, transfer explants for shoot elongation on MS medium devoid of PGRs.
3. Excise in vitro shoots and transfer on MS medium without PGRs for further elongation.
4. After 10 weeks, culture-elongated shoots on MS medium containing 1.0 mg/L BA.

#### 3.4.2. In Vitro Flowering

1. Cut stem sections: 1.5–2 cm long nodal sections with a lateral bud.
2. Culture explants on M1, M2, and M3 media under the similar conditions. Full strength MS medium is inhibitory to in vitro flowering (see Note 1)
3. After 4 weeks of culture initiation on, flower buds develop and become inflorescence apex (see Note 2).
4. Avoid simultaneous reduction in macro- and microelements of MS culture medium for preventing adverse impact on plant growth and development (see Note 3).
5. After 10 weeks, flowers bloom fully showing differences in petal color (see Note 4; Fig. 2).

### 3.5. Acclimatization

1. After the senescence of flowers, transfer the plantlets for hardening in jam bottles (15 cm diameter  $\times$  15 cm high), filled with sterile moistened sand: soil mixture (1:1), adjust pH 5.8 with 0.1 M NaOH and 0.1 M HCl.

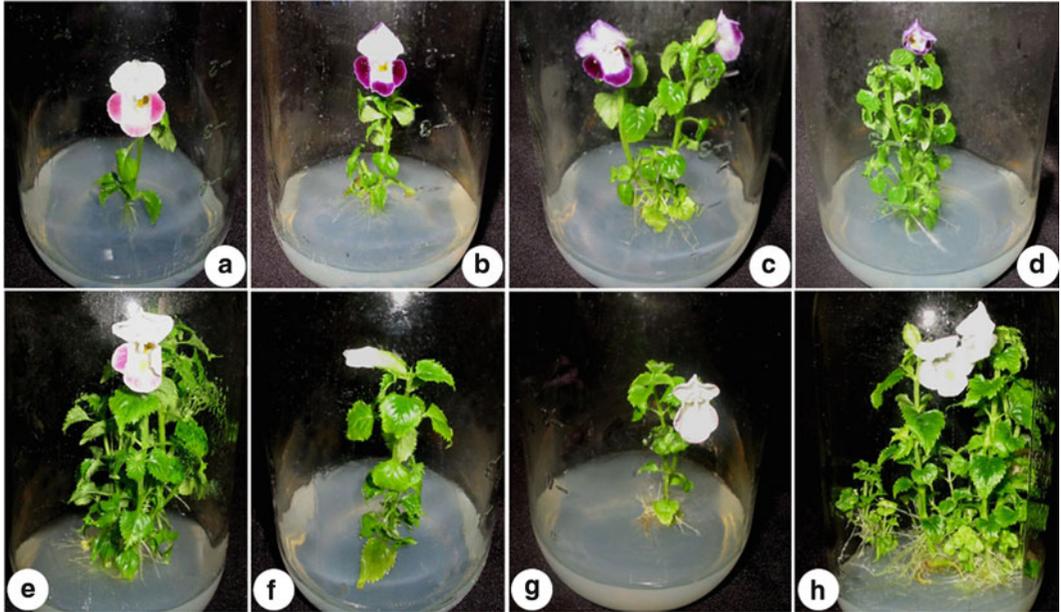


Fig. 2. Somaclonal variation in *Torenia* flower color in vitro. (a, e) *Torenia* flower (purple, control) on M3 after 8 and 10 weeks culture; (b, c) *Torenia* flower in vitro on MS and M1 after 8 weeks culture; (d) Flower *Torenia* in vitro on M2 after 10 weeks culture; (f, g, h) white *Torenia* flower on M3 medium after 7, 8, and 10 weeks culture, respectively.

2. After 5–6 weeks, transfer plantlets to poly bags containing the same potting mixture.
3. Keep them in the greenhouse for acclimatization at 25°C and 80% humidity.

### 3.6. Testing Stability of Flower Color Ex Vitro

1. After observation of flower characters in vitro plants, grow them in the greenhouse until the next flowering to confirm the stability of the variations.

### 3.7. Evaluation of Flower Morphology and Chromosomes

1. Prepare DAPI stain solution according to Table 2.

### 3.8. Flower Morphology

1. Record plant growth and flower morphology by photography from 2 to 10 days after opening of flowers. Take observations of variation in flower phenotypes including color, flower form.

### 3.9. Flow Cytometry Analysis to Determine Ploidy of Somaclones

1. Determination of ploidy with flow cytometer. Excise about 1 cm<sup>2</sup> tissue segments from petals of normal and two somaclones of *Torenia*. Chop petal segments into small pieces with a surgical knife in DAPI solution for releasing and staining the nuclei.

2. Filter DAPI solution containing free nuclei with 20  $\mu\text{m}$  nylon mesh for removing large cell debris. Samples were kept in darkness for 30 min before flow cytometry. All stages of the extraction and staining were performed at 4°C.

#### 4. Notes

1. MS medium inhibits in vitro flowering because of high mineral content, especially N (12).
2. Half-strength MS medium containing  $\frac{1}{2}$  strength macrolelements is suitable for flowering.
3. In half-strength MS or  $\frac{1}{4}$  MS media, simultaneous reduction of both macro- and microelements is not appropriate for plant development. In these media, the C/N ratio is similar to MS medium.
4. White or purple flower color varieties of *Torenia* are successfully developed from the violet variety on M3 medium. The endopolyploidy level, the frequency of cells with 2C, 4C, 8C, and 16C DNA content, showed no difference in both violet and white lines (see Fig. 3).

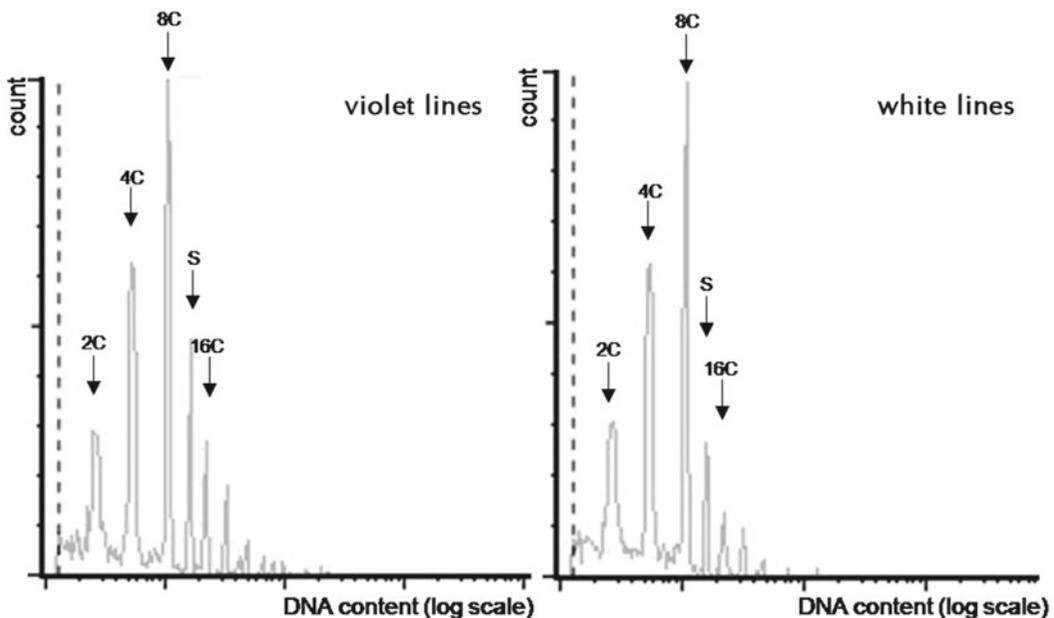


Fig. 3. DNA ploidy distribution in differentiated leaves measured by flow cytometry. Violet lines (left) and white lines (right). S: internal standard. No significant differences in the frequency of endopolyploid cells were found between violet and white lines.

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## Production of Pathogen-Free Horticultural Crops by Cryotherapy of In Vitro-Grown Shoot Tips

Chaohong Feng, Renrui Wang, Jingwei Li, Biao Wang, Zhenfang Yin, Zhenhua Cui, Baiquan Li, Wenlu Bi, Zhibo Zhang, Mingfu Li, and Qiaochun Wang

### Abstract

Horticultural crops are economically valuable for sustainable agricultural production. Plant diseases caused by Pathogens including virus, phytoplasma and bacterium have been a great threat to production of horticultural crops. The efficient use of pathogen-free plant materials has overcome the menace of plant diseases and has sustained crop production. Cryotherapy of shoot tips, a novel application of cryopreservation technique, has become a new plant biotechnology tool for plant pathogen eradication. When compared with the traditional methods, cryotherapy of shoot tips produces high frequency of pathogen-free plants, which is independent of shoot tip size and cryogenic methods. Cryotherapy of shoot tips has six major steps to produce pathogen-free plants: (1) introduction of infected plant materials into in vitro cultures; (2) excision of shoot tips; (3) cryotherapy; (4) post-culture for plant regeneration; (5) indexing of pathogens in regenerated plants after cryotherapy; and (6) establishment of pathogen-free nuclear stock plants. The key steps 2, 3, and 4 are similar to cryopreservation, and play a major role in obtaining high pathogen eradication frequency.

**Key words:** Bacterium, Cryopreservation, Cryotherapy, Horticulture, Pathogen, Phytoplasma, Shoot tips, Virus

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## 1. Introduction

### **1.1. Significance of Pathogen-Free Production of Horticultural Crops**

Horticultural plants including fruits, berries, grapevines, citrus, banana, tubers, vegetables, flowers, and ornamentals are economically important in agricultural production. Tuber crops such as potato, sweetpotato, and cassava serve as food security in many developing countries (1, 2). Fruits and vegetables are primary source in providing essential minerals and vitamins for human health, while flowers and ornamentals are used for all occasions (3). Over the last two decades, horticultural market has expanded rapidly worldwide (4).

The sustainable horticultural crop production has been under continued threat from plant diseases caused by virus, phytoplasma and bacterium (5, 6). Almost every plant developmental stage is affected by plant diseases (7–9), resulting in considerable yield losses, e.g., 30–50% by *Potato virus Y* (PVY) (10), 100% by phytoplasma-induced papaya dieback (8), and 100% by citrus Huanglongbing (HLB) (9), a bacterial disease. A great number of examples of yield losses caused by viruses and phytoplasma have been reviewed (5, 6). In the worst cases, crops are totally annihilated by pathogens, e.g., *Citrus tristeza virus* (CTV) destroyed 100 million citrus trees worldwide (11) and citrus HLB (9) killed hundreds of thousands of trees in Brazil since its first report appeared in 2005 (12).

Most of the horticultural crops are vegetatively propagated either by grafting or rooting of cuttings for maintaining the unique traits of the cultivars. Thus, pathogens are prone to transmit from the infected stock plants to the propagating materials (7). Unlike those by fungi, diseases induced by virus and phytoplasma cannot be cured by chemicals (5, 13). Therefore, the usage of pathogen-free plant material is an ideal approach to prevent damage by these diseases (1, 5, 7, 13–15).

It is well known since long that viruses are unevenly distributed in plants. The virus concentration increases as the distance increases from the apical dome and shoot meristem of virus-infected plants is frequently devoid of viral particles or contains very low viral concentration (16, 17). Based on this concept, Morel (18) was the first to obtain *Dahlia* virus-free plants, using aseptic culture of meristem. At the same time, Holmes (17) obtained virus-free *Dahlia* plants by grafting meristems upon healthy rootstocks. Morel and Martin (19, 20) successfully introduced in vitro meristem culture to produce virus-free *Dahlia* and potato (*Solanum tuberosum*) plants. These results generated a great interest in the production of virus-free plant material. Since then, various methods have been developed for virus eradication, such as meristem culture, micrografting, thermotherapy, and thermotherapy followed by meristem culture (21, 22). Phytoplasmas and vasculature-limited obligate bacteria inhabit only the plant vascular tissues and unable to invade meristematic tissues (23, 24). Thus, the traditional methods mentioned above are also valid for eradication of them.

Production and usage of virus-free plant materials in practical production of fruit trees began as early as 1950s. In USA, an International Project-2 (IR-2) was initiated in 1954 with the main objective to produce virus-free plant materials of important deciduous fruit trees, and deliver virus-free clones to commercial nurseries in both USA and Canada (25). In 1992, the program was renamed National Research Support Project-5 (NRSP-5). In the last 60 years, NRSP-5/IR-2 was the major source for the production of virus-free deciduous fruit trees in USA and worldwide. In England, virus-free sweet cherry cultivars were released by the East Malling Research Station to the growers since 1953 (5). By the

middle of 1960s, several virus-free important fruit tree scions and rootstocks had been used commercially. In 1965, East Malling and Long Ashton initiated a joint EMLA scheme (26) with an objective. To further promote research, production and use of virus-free fruit trees. Until now, virus-free plant materials have been widely used in almost all vegetatively propagated important horticultural crops, such as tuber crops (27, 28), fruit trees (25, 29), grapevines (30), citrus (31), strawberry (32) and ornamentals (33).

A great number of field trials have proven that the usage of virus-free plant materials benefit sustainable production of horticultural crops. Sweetpotato seed tubers free from *Sweetpotato feathery mottle virus* (SPFMV) and *Sweetpotato chlorotic stunt virus* (SPSCV) enhanced 20–62 and 11–33% total yield and marketable tuber yield (individual tubers >100 g), compared to virus-infected seed tubers (34). Similarly, berry yield increased by 65.3 and 26.1% when vines free of *Grapevine leafroll-associated virus* (GLRaV) and *Grapevine fanleaf virus* (GFLV) were used (35). Furthermore, grapevine berry quality produced from virus-free plants was superior over that from virus-infected plants, as determined by berry weight, Ravaz index, sugar content, and total polyphenols (35). Healthy *Alstroemeria* plants produced more vigorously vegetative growth and better flowers than those infected with *Alstroemeria mosaic potyvirus* (AIMV) (36). Pathogen-free plant materials were beneficial for high yield and high quality production of horticultural crops (5, 6).

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## 2. Cryotherapy of Shoot Tips for Pathogen Eradication

Cryopreservation is termed as the storage of living cells, tissues or organs at an ultra-low temperature, usually in liquid nitrogen (LN,  $-196^{\circ}\text{C}$ ) and has been considered an ideal means for long-term storage of plant germplasm (37–39). Cryotherapy of shoot tips requires a short treatment of shoot tips in LN. It is a novel application of cryopreservation techniques to pathogen eradication from plants (40–42).

### 2.1. Development of Cryotherapy Technique

Sakai was the first who (43) reported that very hardy mulberry twigs prefrozen at  $-30^{\circ}\text{C}$  survived immersion in LN after dehydration mediated by extra organ freezing. Based on this study, a classic freezing technique, i.e. two-step freezing, had been developed for cryopreservation of a number of plants during 1960–1980 (37). Since 1990s, new cryogenic techniques, such as encapsulation-dehydration, encapsulation-vitrification, droplet-vitrification and vitrification have been introduced to cryo-store plants (37, 38, 44–46). These new techniques turned out to be cost effective by avoiding usage of expensive programmable freezers; and applicable to shoot tips and somatic tissues (37–39). To date, they have been

successfully applied to most of important horticultural vegetatively propagated crops (37, 38, 44–47).

As mentioned earlier, meristematic cells are usually free from virus. Following cryopreservation, only cells locating at the top layers in apical dome are able to survive, while other cells are killed. Therefore, cryopreservation of shoot tips might be considered as a potential method for virus eradication (48). Based on these assumptions, Brison et al. (48) tested for the first time effects of cryopreservation by vitrification of shoot tips on virus elimination, using a *Prunus* rootstock cv. Fereley-Jaspi (R) infected with *Plum pox virus* (PPV). They found that about 50% of plantlets regenerated from cryopreserved shoot tips were virus-free, while meristem culture gave only 19% virus-free plantlets. Five years later, working on a dessert banana (*Musa*) cv. Williams (AAA, Cavendish subgroup), which were double-infected with *Banana streak virus* (BSV) and *Cucumber mosaic virus* (CMV), Helliott et al. (49) reported that cryopreservation of shoot tips resulted in 30% and 90% plantlets free of CMV and BSV, respectively. Soon after, with an attempt to eliminate *Grapevine virus A* (GVA) from grapevine (*Vitis vinifera* L.), Wang et al. (50) found that virus-free frequency was 97% in plantlets regenerated from cryopreservation. They further confirmed that virus elimination occurred only through freezing step, while any other steps of cryopreservation procedure were not able to eliminate virus. *Potato leafroll virus* (PLRV) and PVY are two of the most damaging viruses in potato production worldwide (14). Wang et al. (51) demonstrated that both PLRV and PVY could be efficiently eliminated by cryotherapy of shoot tips, with 83–86 and 91–95% of virus-free frequencies obtained for the former and latter. Since then, cryotherapy of shoot tips has been successfully applied to eradication of 11 different viruses (40–42, 52). Until now, cryotherapy of shoot tips has been successfully applied to eliminate viruses, phytoplasmas (53) and the vasculature-limited obligate bacteria (see Table 1) (54). Therefore, cryotherapy of shoot tips has recently been recognized as a new plant biotechnology for pathogen eradication (40–42, 52).

## **2.2. Mechanism Involved in Cryotherapy for Pathogen Eradication**

Several studies on histology, ultra-structure of cells, and localization of plant pathogens in shoot tips have been conducted (48, 53, 55–58) to understand the mechanism of eradication of plant pathogens by cryotherapy of shoot tips.

Histological and ultrastructural studies showed that all cells in the shoot tips had dense-stained nucleoli and well-preserved cytoplasm (see Fig. 1) (48, 53, 55–58), and contained an abundance of organelles such as proplastids and mitochondria (see Fig. 2) (53, 55, 57, 58). Cells in the meristematic dome and the youngest leaf primordia 1 and 2 (LP 1 and 2) were small in size, contained small vacuoles and had a high nucleo-cytoplasm ratio (see Fig. 2), while the size of cells and vacuoles increased, and the

**Table 1**  
**Comparison of cryotherapy of shoot tips (Cryo) and meristem culture (Meri-cult) for their efficiency in pathogen eradication**  
**(With kind permission of Copyright Clearance Center of John Wiley and Sons, Wang et al. (40))**

Plant	Pathogen <sup>a</sup>	Plant regeneration from shoot tips (%)		Pathogen-free regenerants (%)		Ref.
		Meri-cult	Cryo	Meri-cult	Cryo	
Banana	CMV	100	76	4	34	[49]
Banana	BSV	100	76	52	90	[49]
Grapevine	GVA	75	60	12	96	[56]
Potato	PLRV	55	87	56	85	[57]
Potato	PVY	55	87	62	93	[57]
<i>Prunus</i> hybrid	PPV	85	50	19	50	[55]
Raspberry <sup>b</sup>	RBDV	60	30	0	35	[60]
Sweet orange	HLB	69	85	25	98	[59]
Sweetpotato <sup>c</sup>	SPCSV	100	87	100	100	[54]
Sweetpotato <sup>c</sup>	SPFMV	100	87	10	100	[54]
Sweetpotato <sup>c</sup>	SPCSV +	100	87	7	100	[54]
Sweetpotato <sup>d</sup>	SPFMV SPLL	100	85	10	100	[53]

<sup>a</sup>CMV *Cucumber mosaic virus*; BSV *Banana streak virus*; GVA *Grapevine virus A*; PLRV *Potato leafroll virus*; PVY *Potato virus Y*; PPV *Plum pox virus*; RBDV *Raspberry bushy dwarf virus*; HLB *Huanglongbing*; SPCSV *Sweetpotato chlorotic stunt virus*; SPFMV *Sweetpotato feathery mottle virus*; SPLL *Sweetpotato little leaf phytoplasma*

<sup>b</sup>Shoots were subjected to thermotherapy followed by cryotherapy of the excised shoot tips

<sup>c</sup>Shoot tips size 1.5 mm

<sup>d</sup>Shoot tip size 1.0 mm

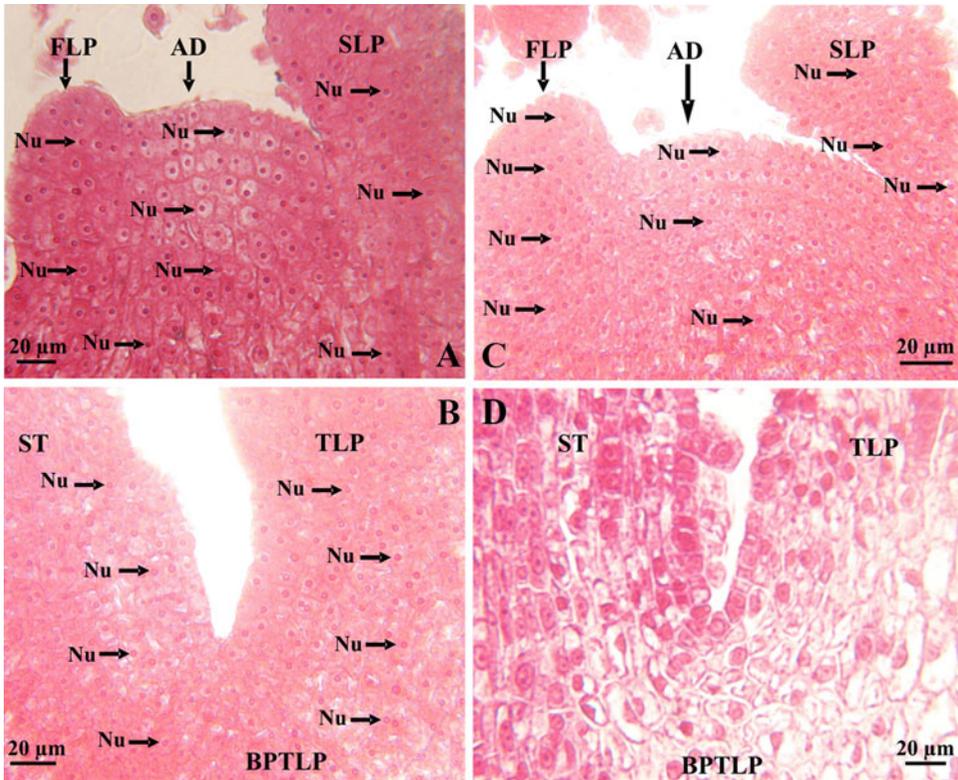


Fig. 1. Longitudinal sections of sweetpotato shoot tips observed under light microscope. Sections were stained with safranin for recognition of living or surviving cells by intensive purple staining of nucleoli (Nu). (a) Apical part of a nontreated (control) shoot tip with the first (youngest) and the second leaf primordia (FLP and SLP, respectively). (b) Basal part of the nontreated shoot tip in conjunction with the third leaf primordium (BPTLP). (c) Apical part of a cryo-treated shoot tip, 1 day after post-culture. (d) Basal part of a cryo-treated shoot tip in conjunction with BPTLP 1 day after post-culture. AD apical dome of the meristem. (With kind permission of Copyright Clearance Center of John Wiley and Sons, Wang et al. (53)).

nucleo-cytoplasm ratio decreased with increasing distance from the apical dome (see Fig. 2) (53, 55). Lethal damage caused by freezing in LN was usually observed in cells with big vacuoles and low nucleo-cytoplasm ratio, while leaving alive cells with small vacuoles and high nucleo-cytoplasm ratio (53, 55, 57, 58). Thus, following cryotherapy, cells in the upper part of the apical dome and the youngest LP are able to survive, while those in the lower part of the apical dome and more differentiated LP are killed (see Fig. 1) (49, 53, 55–58).

Virus localization in shoot tips demonstrated that SPFMV can invade the fourth LP and older tissues, but not the younger LP (1–3) and apical dome, whereas SPCSV can reach only the fifth LP (see Fig. 3) (57). Distribution pattern of these viruses was similar irrespective of whether the plants were single-infected or coinfecting (57). *Sweetpotato little leaf* (SPLL) phytoplasma was observed only in sieve elements of the third LP, but not in those of the youngest LP 1 and 2, and apical dome (see Fig. 4) (53).

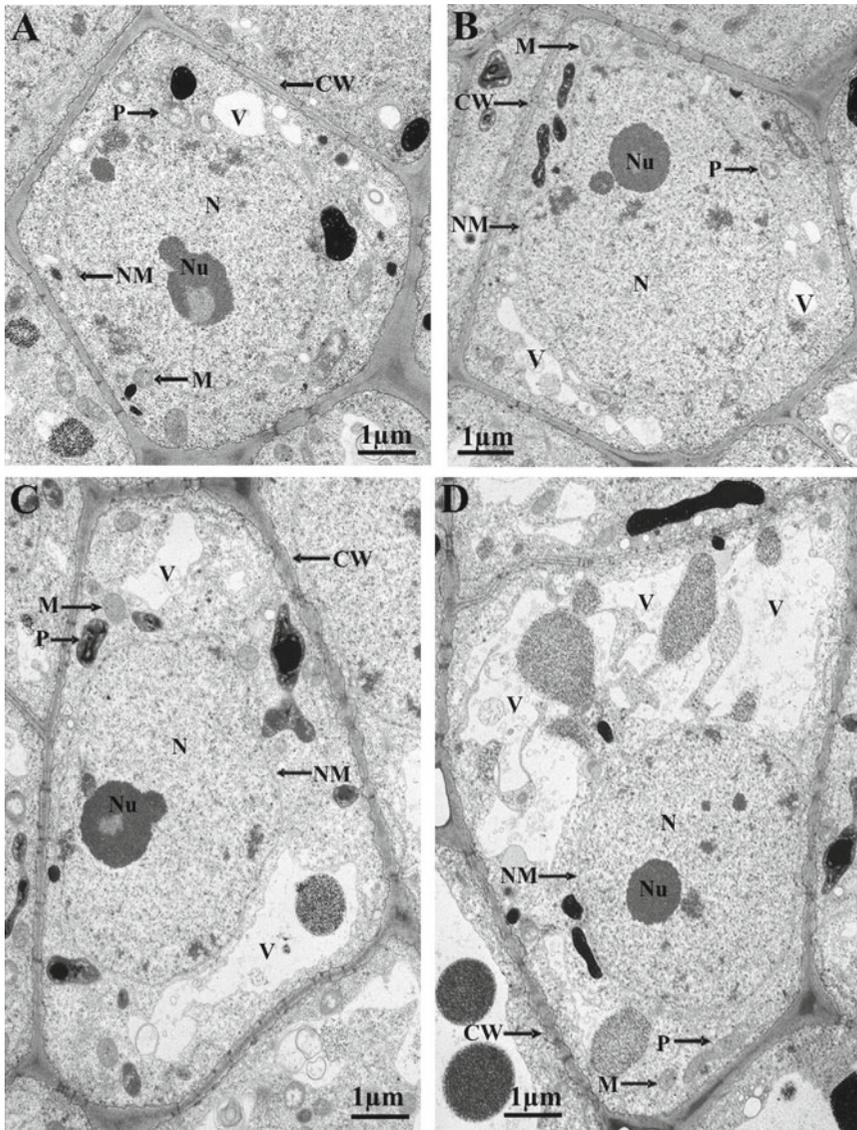


Fig. 2. Transmission electron microscopy of cells in different parts of sweetpotato shoot tips. (a) Top layer of apical dome; (b) First leaf primordium (LP1); (c) Basal part of meristem in conjunction with the third leaf primordium (LP3); and (d) LP3. CW cell wall; N nucleus; NM nucleolus; M mitochondria; V vacuole; P proplastid. (With kind permission of Copyright Clearance Center of John Wiley and Sons, Wang et al. (53)).

Thus, the localization of plant pathogens in shoot tips before cryotherapy and determination of cell surviving pattern in shoot tips after cryotherapy revealed the mechanism of efficient elimination of plant viruses, phytoplasmas, and vasculature-limited obligate bacteria (see Fig. 5) (40–42). However, some viruses can infect meristematic cells, e.g., CMV in banana shoot tips (59) and *Raspberry bushy dwarf virus* (RBDV) in raspberry (*Rubus idaeus*) shoot tips (58). In such cases, cryotherapy of shoot tips alone failed to eliminate

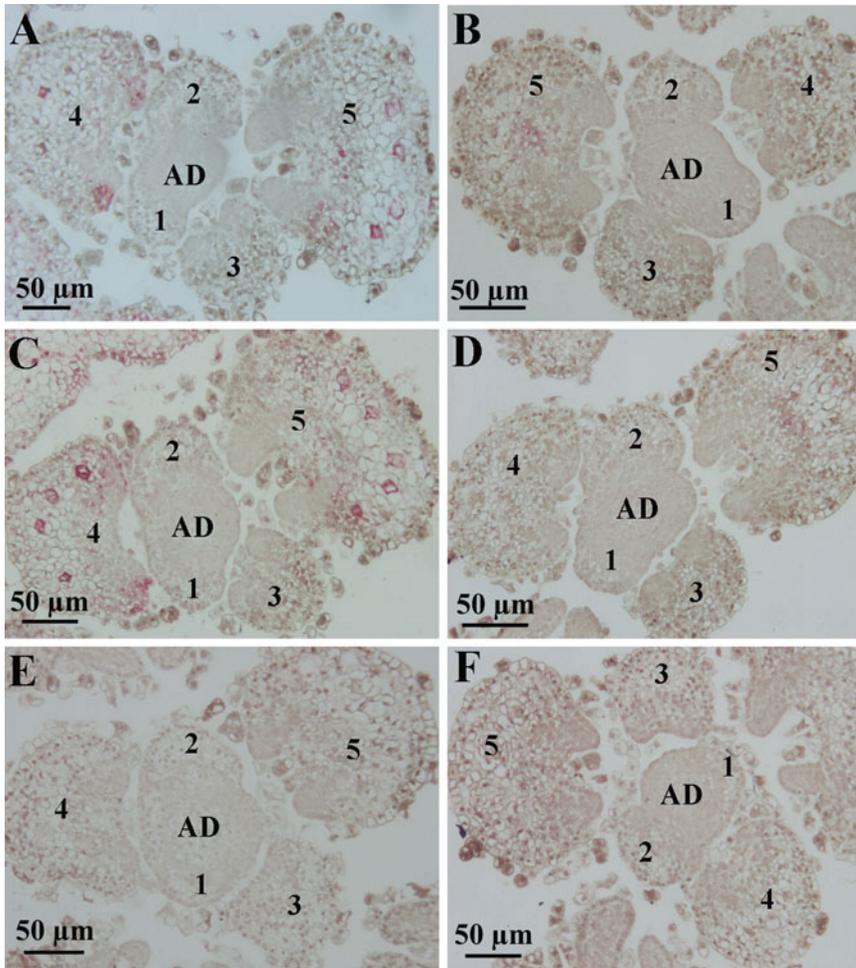


Fig. 3. Immunohistochemical localization of SPFMV and SPCSV in cross-sections shoot tips of sweetpotato line 199004.2. Sections **a**, **c**, and **e** were stained with antibodies to SPFMV, whereas sections **b**, **d**, and **f** were stained with antibodies to SPCSV. Virus-infected cells are detected by purple staining in **a–d**, whereas no signals are detected in tissues of virus-free shoot tips (**e** and **f**). The shoot tips in **a** and **b** were infected only with SPFMV and SPCSV, respectively. Views **c** and **d** show consecutive sections cut from a shoot tip coinfecting with SPFMV and SPCSV. AD apical dome of meristem; 1–5 = leaf primordium 1–5, respectively. (Ding et al. (54), With kind permission of Copyright Clearance Center of Elsevier).

them from infected plants; thermotherapy followed by cryotherapy of shoot tips can significantly enhance virus eradication (58).

**2.3. Characteristics of Shoot Tip Cryotherapy for Pathogen Eradication**

Compared to the more traditional methods such as meristem culture, major characteristics involved in cryotherapy of shoot tips for pathogen eradication are summarized as follows:

1. Cryotherapy of shoot tips consistently results in high frequency of pathogen eradication. Indeed, from the first report on virus eradication by cryotherapy, Brison et al. (48) found that a much higher frequency of virus elimination was obtained by

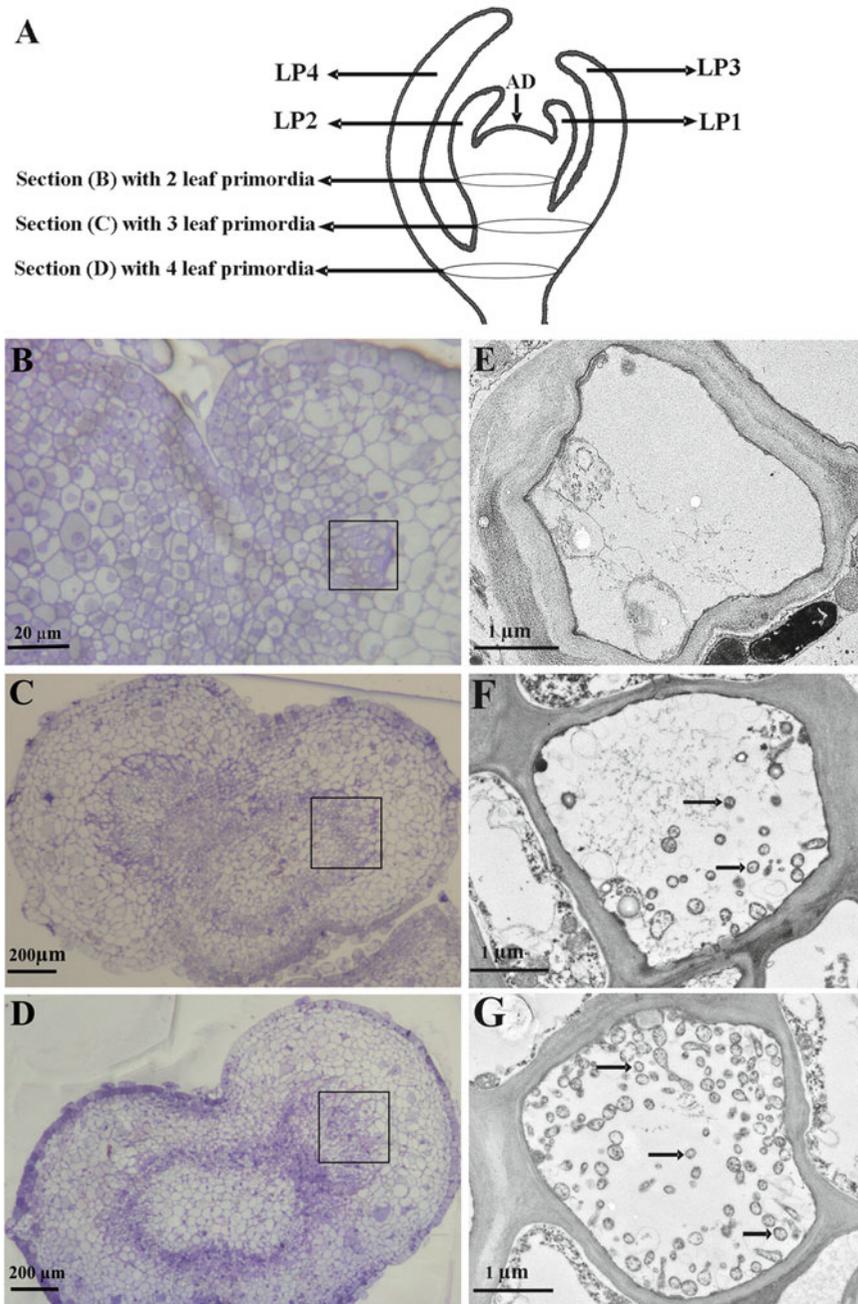


Fig. 4. Vascular tissues and phytoplasma in cross-sections of sweetpotato shoot tips. (a) Schematic drawing of the shoot tip with four leaf primordia (LP1–LP4) and positions of the sections studied. (e–g) Ultra-thin sections for transmission electron microscopy of sweetpotato little leaf phytoplasma in the vascular tissues that are boxed in the corresponding light microscope image to the left (b–d, respectively). Phytoplasmas, as indicated by arrows in f and g, were observed in the sieve elements at the basal parts of the shoot tips (c, d), but not in the sections cut closer the apical dome (b, e). (With kind permission of Copyright Clearance Center of John Wiley and Sons, Wang et al. (53)).

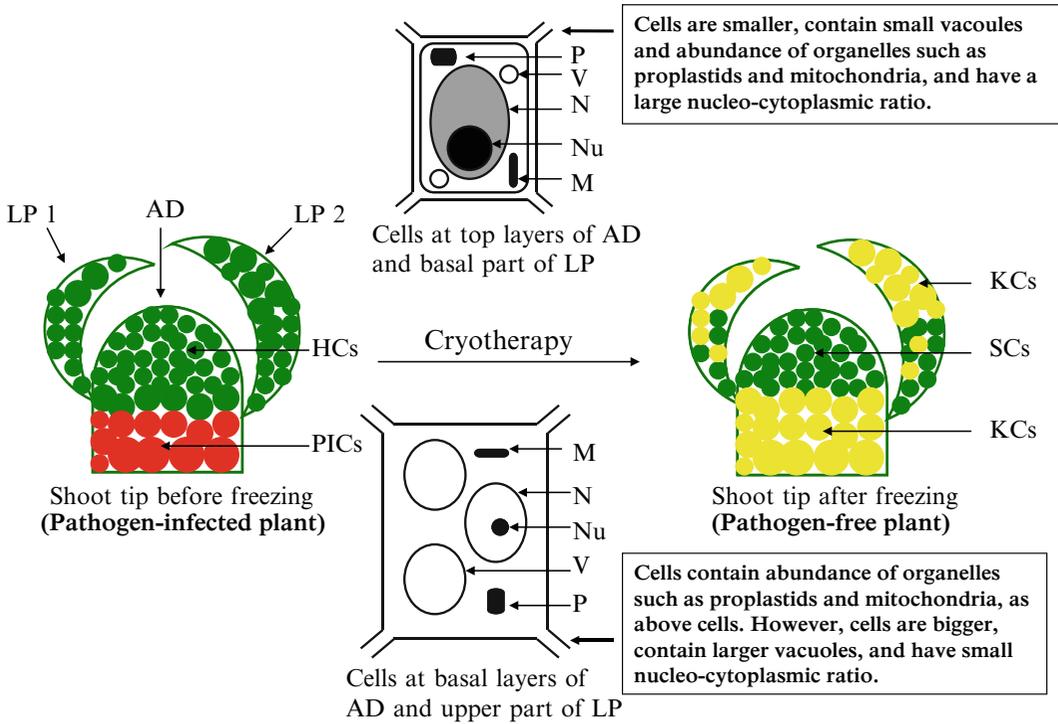


Fig. 5. An action model of plant pathogen eradication by cryotherapy of shoot tips. Cells in the upper part of the apical dome and the youngest leaf primordia (1 and 2) are small and isodiametric in shape, and possess a large nucleo–cytoplasmic volume ratio. Vacuoles are small and scattered in the cell. The protoplasm contains a large number of cell organelles such as proplastids, mitochondria, Golgi apparatuses, and ribosomes associated with the endoplasmic reticulum. The sizes of cells and vacuoles increase, while the nucleo–cytoplasmic ratio decreases, with increasing distance from the apical dome. *AD* apical dome; *HC* healthy cells; *KC* killed cells; *LP 1* leaf primordium 1; *LP 2* leaf primordium 2; *M* mitochondria; *N* nucleus; *Nu* nucleolus; *P* proplastid; *PIC* pathogen-infected cells; *SC* surviving cells; *V* vacuoles (With kind permission of Copyright Clearance Center of Elsevier, Wang et al. (41)).

cryotherapy (50%) than by meristem culture (19%). Up to date, results obtained from various laboratories showed that cryotherapy of shoot tips consistently produced higher eradication frequencies of viruses (48–51, 57), phytoplasmas (53), and vasculature-limited obligate bacteria (54) than the more traditional methods (see Table 1). The eradication frequencies obtained by cryotherapy of shoot tips were almost doubled with BSV (49) and 14 times with coinfection by SPFMV and SPCSV (57) of those from the traditional methods (see Table 1).

2. The frequency of virus eradication is independent of size of shoot tips. It is well known that size of meristem is a key factor in determining the virus-free frequency in meristem culture. Virus-free frequency is negatively related with the size of meristem. Therefore, care must be taken to excise meristems that are small enough (usually 0.2–0.4 mm in size). Excision of such

small meristems is a skillful task, and culture of them makes plant regeneration difficult, because the regeneration ability is positively proportional to the size of the meristem (21).

When cryotherapy is applied to plant pathogen eradication, size of shoot tips does not affect pathogen-free frequency. With PPV, cryopreserved shoot tips ranging from 0.5 to 2 mm in size produced similar high virus-free frequencies. In contrast, virus-free frequencies obtained by shoot tip culture markedly decreased when size of shoot tips increased from 0.5 to 2.0 mm (48). Similar results were found with GVA (50), SPCSV (57), SPFMV (57), and citrus HLB (54).

3. The frequency of virus eradication is not affected by cryotherapy methods. When compared the effects of two cryotherapy treatments on GVA elimination, Wang et al. (50) found that encapsulation-dehydration and vitrification produced the same virus-free frequencies. With PLRV and PVY, virus-free frequencies obtained by encapsulation-dehydration, encapsulation-vitrification, and droplet were also the same (51).
4. Plant regeneration produced by cryotherapy is generally lower than that by shoot tip culture, but high enough to be acceptable. Most of the previous studies on comparison of pathogen elimination between cryotherapy of shoot tips and shoot tip culture clearly demonstrated that plant regeneration from cryotherapy-treated shoot tips was significantly lower than that from shoot tip culture (48–50, 53, 57). In two studies (51, 54), cryo-treated shoot tips seemed to produce higher regrowth rates (87 and 85%) than those (55 and 69%) of shoot tip culture. However, these results were not comparable, because the size of shoot tips used for cryotherapy was much bigger (1.0–1.5 mm) than those (0.2–0.3 mm) for shoot tip culture (51, 54). Average regeneration rates calculated from previously comparable 8 reports were 77.8% for shoot tip culture and 59.4 for cryotherapy (40), which is still considered to be high and acceptable in studies aiming at pathogen eradication.
5. Cryotherapy does not require longer period of time for production of pathogen-free plants than the traditional methods. Indeed, all previous studies demonstrated that time period required by cryotherapy and shoot tip culture was almost the same (48–51, 53, 54, 57). Working on the production of PLRV- and PVY-free plants, Wang et al. (51) found that time period of the whole procedure required by cryotherapy and shoot tip culture was similar (55 and 59 days, respectively), while thermotherapy and thermotherapy followed by meristem culture needed about 87 days to complete the whole procedure.
6. A genotype-specific response is common, which is similar to the traditional methods. As discussed earlier, cryotherapy of shoot

tips for pathogen elimination is based on cryopreservation techniques (40–42). Therefore, genotype-specific response to cryotherapy protocols is common, which is the main limitation for wider application of the available protocols to all genotypes or cultivars of the same species. Similar limitations are also experienced with the more traditional methods for pathogen eradication (21, 22, 28).

### 3. Steps to Produce Pathogen-Free Plants by Shoot Tip Cryotherapy

Let’s take encapsulation-dehydration as an example to demonstrate the major steps involved in this cryotherapy procedure for production of pathogen-free plants (see Fig. 6).

#### 3.1. Step 1. Introduction of Infected Plant Materials into In Vitro Cultures

By tissue culture techniques, various explants like single nodes, buds, shoot tips, and leaves can be used to establish in vitro stock cultures from infected plants (50, 51, 58). Explants are surface-sterilized and then cultured on a suitable medium for the

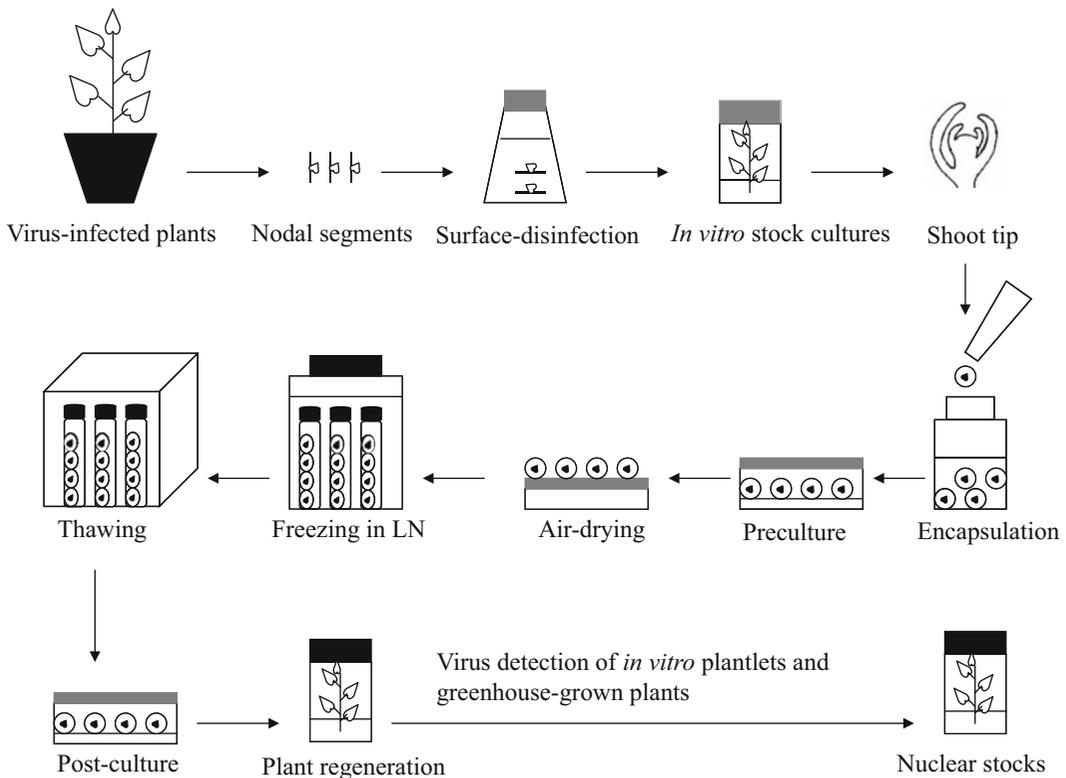


Fig. 6. Main steps involved in production of pathogen-free plants by encapsulation-dehydration cryotherapy of shoot tips.

establishment of *in vitro* stock cultures—a procedure commonly used in plant tissue culture.

### **3.2. Step 2. Excision of Shoot Tips**

The shoot tip size does not affect pathogen-free frequency. However, it influences the plant regeneration rate following cryotherapy (48, 53, 54). Therefore, a suitable size of shoot tips for specific plant species or genotype should be excised. Shoot tips ranging from 0.5 to 2.0 mm long can be used, depending on genotypes (48–51, 53, 57).

### **3.3. Step 3. Cryotherapy**

As stated earlier, cryotherapy is based on cryopreservation. Therefore, all procedures involved in cryotherapy are similar to Cryopreservation and however, they differ that a brief LN treatment is given for cryotherapy. Cryopreservation protocols for all important horticultural crops are available in several books (44–46). In some cases, well-established cryopreservation protocols may require modification depending on the genotype (40, 41, 53, 57).

### **3.4. Step 4. Post-Culture for Plant Regeneration**

Actually, this step is similar to cryopreservation. However, great attention should be given to direct shoot regrowth without intermediate callus formation, which otherwise may cause somaclonal variation (37, 60). For some plant species like citrus, which are difficult-to-root, *in vitro* grafting can be used to obtain regeneration of whole plants (54).

### **3.5. Step 5. Indexing of Pathogens in Plants Regenerated from Cryotherapy**

Specific pathogen(s) can be indexed in plants regenerated following cryotherapy, using appropriate reliable methods such as enzyme-linked immunosorbent assay (ELISA) for virus coat protein, reverse transcription-polymerase chain reaction (RT-PCR) for virus RNA, western blotting for virus movement protein, polymerase chain reaction (PCR) for phytoplasma and bacterium (7, 61). Molecular marker-based methods have proven to be superior over serological methods like ELISA (7, 13). Indexing of viruses by *in vitro* or *in vivo* grafting upon indicator plants is a reliable method (7, 13) and however, takes longer time than other methods.

To ensure healthy status of the plants from cryotherapy, indexing of pathogen(s) should be carried out at least twice: *in vitro* stage and in the greenhouse stage (49, 50, 53, 54, 57), according to an internationally accepted indexing practice of different types of pathogens.

### **3.6. Step 6. Establishment of Pathogen-Free Nuclear Stock Plants**

Plants originated from cryotherapy and free of specific pathogen(s) are considered as basic nuclear stock plants, and grown in containers filled with sterile medium and maintained in the screen house. Each plant must be tested for specific pathogen(s) and checked for its true-to-typeness. Plants that meet the above-mentioned requirements can be certified as pathogen-free nuclear stock plants and used in propagation system of pathogen-free plants (13).

## **4. Production of PLRV and PVY Virus-Free Plants by Shoot Tip Cryotherapy (51)**

### **4.1. Materials**

#### *4.1.1. General Requirements*

For Cryotherapy

1. Screw capped bottles (100 mL).
2. Cryotubes (1.8 mL).
3. Liquid nitrogen (LN).
4. Pipette (1 mL).
5. Standard tissue culture facilities.
6. Sterile Petri dishes (9 cm in diameter).
7. Sterilized tips (1 ml).
8. Sterile distilled water.
9. Sterile Whatman paper (9 cm in diameter).
10. Tap water.
11. Water bath (set at 40°C).

For Virus Detection by ELISA

1. Eppendorf tubes (1.5 mL).
2. ELISA plate washer.
3. ELISA plate reader.
4. An incubator (set up at 37°C).
5. Pipette (200 µL).
6. Eight-channel pipette (200 µL for each).
7. 96-well microtiter plates.

#### *4.1.2. Chemicals, Plant Growth Regulators, and Antibodies*

For Cryotherapy

1. 6-Benzylaminopurine (BAP).
2. Calcium chloride.
3. 75% ethanol.
4. Gelrite.
5. Gibberellic acid 3 (GA<sub>3</sub>).
6. Glycerol.
7. Hypochlorite (commercial bleach solution).
8. Murashige and Skoog medium (MS) (62).
9. Sodium alginate.
10. Sucrose.

For Virus Detection by ELISA

1. ELISA kit for PLRV (Loewe Biochemica GmbH, Germany or any other companies available).
2. ELISA kit for PVY (Loewe Biochemica GmbH, Germany or any other companies available).

#### 4.1.3. Media and Solutions

1. Basic medium (BM): MS containing 30 g/L sucrose and 2.6 g/L Gelrite (pH = 5.8).
2. Calcium chloride solution: liquid MS medium containing 0.1 M calcium chloride, 2 M glycerol and 0.4 M sucrose, pH 5.8.
3. 1% hypochlorite solution: commercial bleach solution diluted to 1:10 (v:v) with tap water.
4. Recovery medium: BM added with 0.4 mg/L BAP and 1 mg/L GA<sub>3</sub>, pH 5.8.
5. Sodium alginate solution: 0.4 M sucrose, 2 M glycerol and 2.5% (w/v) sodium alginate made up in liquid MS medium, pH 5.8.
6. 0.25 M sucrose preculture medium: MS medium containing 85.5 g/L sucrose, 2.6 g/L Gelrite, pH 5.8.
7. 0.5 M sucrose preculture medium: MS medium amended with 171 g/L sucrose, 2.6 g/L Gelrite, pH 5.8.
8. 0.75 M sucrose preculture medium: MS medium added with 256.5 g/L sucrose, 2.6 g/L Gelrite, pH 5.8.

## 4.2. Methods

### 4.2.1. Establishment of In Vitro Stock Cultures

1. Incubate PLRV- and PVY-infected potato (*S. tuberosum*) seed tubers that have been stored at 4°C for 2–3 months, at room temperature until shoots (about 4 cm in length) are produced.
2. Single nodal segments (0.5 cm in length) are removed from 4 cm long shoots.
3. Use them as explants for the establishment of in vitro stock cultures.
4. Wash explants with tap water for 30 min
5. Surface disinfect explants with 75% ethanol for 30–60 s, followed by 1% NaClO for 8–10 min and wash three times in sterile water.
6. Culture explants on a basic medium (BM) composed of Murashige and Skoog medium (MS, 62) supplemented with 30 g/L sucrose, solidified with 2.6 g/L Gelrite. The pH was adjusted to 5.8 prior to autoclaving at 121°C for 20 min.
7. Maintain stock cultures at 22 ± 1°C under a 16-h photoperiod with light intensity 50 μEs/m provided by cool-white fluorescent tubes.
8. Subculture in vitro cultures on fresh culture medium once every 4 week interval.

### 4.2.2. Excision of Shoot Tips and Encapsulation

1. Excise shoot tips (1.0–2.0 mm in size) (see Note 1) from 4-week-old stock cultures.
2. Suspend shoot tips in the sodium alginate solution prepared in liquid MS medium.

3. Drop a mixture of sodium alginate solution with shoot tips with a sterile pipette (1 ml) into 0.1 M CaCl<sub>2</sub> solution
4. Leave them for 20 min to form beads, each being about 4–5 mm in diameter and containing one shoot tip.

*4.2.3. Preculture*

1. Surface dry beads rapidly by blotting on sterile Whatman paper.
2. Preculture beads stepwise in 0.25, 0.5, and 0.75 M sucrose solution, with each step for 1 day (see Note 2).

*4.2.4. Dehydration*

1. Surface dry beads by blotting on sterile Whatman paper.
2. Transfer beads on new sterile Whatman paper placed on sterile Petri dish (9 cm in diameter), with 10 beads per each Petri dish.
3. Dehydrate beads by air drying in laminar hood at room temperature and moisture to reduce water content of the beads to about 20 % (see Note 3).

*4.2.5. Cryotherapy*

1. Transfer 10 dehydrated beads into each cryotube (1.8 ml) and immerse the cryotubes directly in LN for 1 h.
2. Remove cryotubes from LN and rapidly thaw at 40°C for 3 min.
3. Culture thawed beads on recovery medium.
4. Maintain cultures in the darkness at 22 ± 1°C for 3 days, followed by transfer to light for the recovery.

*4.2.6. Plant Regeneration*

1. After 3–5 days of culturing, green tissues appear in cryo-treated shoot tips, indicating signal of survival. Shoot elongation starts from surviving shoot tips within 7 days of culture. Shoots longer than 0.5 cm regenerate after 4–5 weeks of culture.
2. Transfer shoots (>0.5 cm long) onto BM for rooting, and the shoots develop roots within 1–2 weeks. Subculture is done once 4 weeks.

*4.2.7. Virus Detection of Plantlets Regenerated from Cryo-Treated Shoot Tips*

1. After 2 months of rooting, cut plantlets into two parts.
2. Use top part for virus indexing and maintain the basal part on BM.
3. Discard the basal part of the plant when the top part is positive response to virus detection.
4. When the top plant part is negative response to virus detection, continue to maintain the basal part on BM for well-developed root system. These plants are referred as virus-free.
5. Transfer plantlets to soil in the greenhouse.

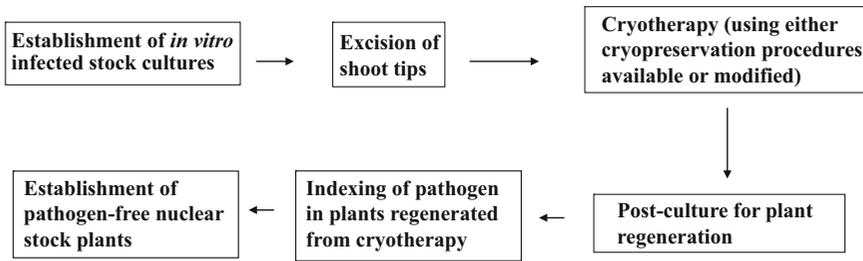


Fig. 7. A flow-chart for production of virus-free plants by encapsulation-dehydration cryotherapy of shoot tips.

6. Test sanitary status of plants to ensure free from viruses after 3 months of establishment in the greenhouse conditions (see Note 4).
7. Use DAS-ELISA test for the evaluation of the sanitary status of the plant sample (see Note 5). All procedures are carried out according to the kit's Instructions provided by Loewe Biochemica GmbH. Absorbance is recorded at 405 nm using ELISA Plate Reader.
8. A flow-chart for production of virus-free plants by encapsulation-dehydration cryotherapy of shoot tips is demonstrated in Fig. 7.

## 5. Notes

1. The frequency rate of virus-free plants produced by cryotherapy is not dependent on shoot tip size (40–42), however may influence survival and regrowth of cryo-treated shoot tips (47, 48, 53). The lower survival and regrowth rates occur when smaller shoot tips are used. For potato, shoot tips 1.0–2.0 mm in size normally give good results (47).
2. Preculture of shoot tips with high sugar concentration is to induce tolerance of shoot tips to dehydration and subsequent freezing in liquid nitrogen. Sucrose is commonly used for this step (47). Sugar concentration used in the preculture medium ranges from 0.3 to 1.0 M (38, 47). For some plant species that are sensitive to high sucrose concentration, stepwise preculture with increasing sucrose concentrations prevents the deleterious effects of direct exposure to high sucrose concentrations (38, 47, 63), finally resulting in high recovery of cryo-treated shoot tips.
3. Two desiccation methods can be employed: (1) dehydration under the air flow of a laminar flow cabinet, and (2) dehydration in sealed containers with dry silica gel (63). Regardless of dehydration methods, the water content level that ensures

highest regrowth following cryopreservation is around 20% on a fresh weight basis (37). This value may vary depending on the species and the type of samples.

4. In general, virus detection should be carried out at least twice; in vitro stage and in greenhouse stage, in order to confirm the sanitary status of the plants (49, 50, 53, 54, 57). Virus detection is performed after 2 months for in vitro plantlets and 3 months for plants established in soil. In some cases, virus titre may be too low to be detected in in vitro plantlets (64). Thus, virus detection using in vivo plants is necessary to ascertain virus-free plants (64, 65).
5. In some cases, virus detection by ELISA is not sufficient enough to confirm sanitary status of the samples tested (65, 66). Thus, more sensitive methods using molecular marker-based analysis, should be employed (65, 66).

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