Mohammad Faisal
Abdulrahman A. Alatar *Editors*

Synthetic Seeds

Germplasm Regeneration, Preservation and Prospects

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Editors
Mohammad Faisal
Department of Botany & Microbiology
College of Science, King Saud University
Riyadh, Saudi Arabia

Abdulrahman A. Alatar
Department of Botany & Microbiology
College of Science, King Saud University
Riyadh, Saudi Arabia

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The book is dedicated to my supervisors

Prof. Mohammad Anis
UGC-BSR Faculty Fellow
Department of Botany
AMU, Aligarh

&

Prof. Sayeed A. Siddiqui
Ex-Chairman
Department of Botany
AMU, Aligarh

Preface

Synthetic or artificial seeds are described as alginate-encapsulated somatic embryos, vegetative buds, or any other micropropagules that can be used as seeds and converted into plantlets after propagating in vitro or in vivo conditions and also sustain the regeneration potential after low temperature storage. Production of synthetic or artificial seeds using micropropagules opens up new vistas in agricultural biotechnology that helped to overcome the challenges that face important economic and medicinal plant species. Encapsulated propagules could be used for in vitro regeneration and mass multiplication at reasonable cost. In addition, these propagules may be use for germplasm preservation of elite plant species and exchange of plant materials between national and international laboratories. Besides, the technology has been successfully utilizing for cryopreservation via encapsulation-dehydration, and encapsulation-vitrification for germplasm storage of elite plant species. Synthetic seeds are reasonably inexpensive to produce and easy to handle, plant, and transport and have great advantages in comparison with traditional in vitro culture methods. The aim of this book is to provide relevant state-of-the-art findings on methods, application, and prospects of synthetic or artificial seeds. Being involved in this area, we comprehend that information on encapsulation and synseed production is still obscure, and there is no single book available on this aspect.

The intended volume comprised several chapters on relevant topics contributed by experts working in the field of plant biotechnology so as to make available a comprehensive treatise designed to provide an in-depth analysis of the subject in question. The book is a compilation of 22 chapters having relevant text, tables, and illustration describing the experimental work on encapsulation and synthetic seeds production, for regeneration, multiplication, germplasm preservation, exchange, and crop improvement in several plant species which will be useful in planning and execution of various experiments smoothly and effectively.

The present book aimed to induce new outlooks to scientists/researcher who are unfamiliar with synthetic seeds and will be very helpful in various present and future researches in different areas of plant biotechnology, cryobiology, molecular biology, plant physiology, and seed biology.

We are extremely thankful to all the contributors who wholeheartedly welcomed our invitation and agreed to contribute chapters to embellish information on synthetic seed production, thus helped in this endeavor.

Riyadh, Saudi Arabia
May 19, 2019

Mohammad Faisal
Abdulrahman A. Alatar

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About the Editors

Mohammad Faisal has over 12 years of research experience in Plant Biotechnology currently working as Associate Professor in the Department of Botany and Microbiology, King Saud University, Riyadh, Saudi Arabia. He has been the recipient of Plant Biotechnologist Award 2017 by SESR, India, and Scientist of the Year Award 2015 by NESA and several National and International Fellowships. He holds membership of several academic bodies/societies and Fellow of the SESR. He has published over 82 research articles, 5 book chapters, and 4 books. He is in the editorial board of the several reputed journals.

Abdulrahman A. Alatar is working as Professor in the Department of Botany and Microbiology, King Saud University, Riyadh, Saudi Arabia. He also serving as consultant for human resource and management in the Ministry of Higher Education, Saudi Arabia. He has been the PI and Co-I of various projects and supervised 3 PhD and 7 MSc students. He published more than 67 scientific articles and 2 books, and his work has been cited in various leading journals.

List of Contributors

Eslam M. Abdel-Salam Department of Botany & Microbiology, College of Sciences, King Saud University, Riyadh, Saudi Arabia

Zishan Ahmad Plant Biotechnology Section, Department of Botany, Aligarh Muslim University, Aligarh, Uttar Pradesh, India

Abdulrahman A. Alatar Department of Botany & Microbiology, College of Sciences, King Saud University, Riyadh, Saudi Arabia

Abubakker Aslam Plant Molecular Biology Laboratory, Department of Botany, Jamal Mohamed College, Tiruchirappalli, India

Asmah Awal Faculty of Plantation and Agrotechnology, Universiti Teknologi MARA, Shah Alam, Selangor, Malaysia

Carla Benelli National Research Council - BioEconomic Institute (CNR-IBE), Sesto Fiorentino, Firenze, Italy

Jean Carlos Bettoni Santa Catarina State University (UDESC), Lages, SC, Brazil CAPES Foundation, Ministry of Education of Brazil, Brasília, DF, Brazil

Remi Bonnart USDA-ARS National Laboratory for Genetic Resources Preservation, Fort Collins, CO, USA

Nandini Boregowda Plant Cell Biotechnology Department, CSIR-CFTRI, Mysuru, Karnataka, India

Francesco Carimi Consiglio Nazionale delle Ricerche, Istituto di Bioscienze e BioRisorse, U.O.S. di Palermo, Palermo, Italy

Angela Carra Consiglio Nazionale delle Ricerche, Istituto di Bioscienze e BioRisorse, U.O.S. di Palermo, Palermo, Italy

Ravishankar Chauhan National Center for Natural Resources, Pt. Ravishankar Shukla University, Raipur, India

Yelda Özden Çiftçi Department of Molecular Biology and Genetics, Gebze Technical University, Kocaeli, Turkey

Fernandes Da Silva Daniel Department of Agricultural, Food and Environmental Sciences (DSA3), University of Perugia, Perugia, Italy

Hamit Ekinci Department of Molecular Biology and Genetics, Gebze Technical University, Kocaeli, Turkey

Ali Ergül Biotechnology Institute, Ankara University, Ankara, Turkey

Mohammad Faisal Department of Botany & Microbiology, College of Sciences, King Saud University, Riyadh, Saudi Arabia

Saikat Gantait Crop Research Unit (Genetics and Plant Breeding), Bidhan Chandra Krishi Viswavidyalaya, Mohanpur, Nadia, West Bengal, India

Biswajit Ghosh Plant Biotechnology Laboratory, Post Graduate Department of Botany, Ramakrishna Mission Vivekananda Centenary College, Kolkata, India

Parvatam Giridhar Plant Cell Biotechnology Department, CSIR-CFTRI, Mysuru, Karnataka, India

R. Gowthami Tissue Culture and Cryopreservation Unit, ICAR-National Bureau of Plant Genetic Resources (NBPGR), Pusa Campus, New Delhi, India

S. K. Moquammel Haque Plant Biotechnology Laboratory, Post Graduate Department of Botany, Ramakrishna Mission Vivekananda Centenary College, Kolkata, India

Tolga Izgu Faculty of Agriculture, Department of Horticulture, University of Ege, Bornova, İzmir, Turkey

Saber Delpasand Khabbazi Biotechnology Institute, Ankara University, Ankara, Turkey

Metin Kocak Faculty of Agriculture, Department of Agricultural Biotechnology, Van Yüzüncü Yil University, Van, Turkey

Dariusz Kulus Faculty of Agriculture and Biotechnology, Department of Environmental Bases of Agriculture and Horticulture, Laboratory of Ornamental Plants and Vegetables Crop, UTP University of Science and Technology, Bydgoszcz, Poland

Suprabuddha Kundu Department of Agricultural Biotechnology, Faculty of Agriculture, Bidhan Chandra Krishi Viswavidyalaya, Mohanpur, Nadia, West Bengal, India

Yatzil León-Romero Natural Products Laboratory, Biotechnology Department, Centro de Desarrollo de Productos Bióticos, Instituto Politécnico Nacional, Yautepec, Morelos, México

Alma Rosa López-Laredo Natural Products Laboratory, Biotechnology Department, Centro de Desarrollo de Productos Bióticos, Instituto Politécnico Nacional, Yautepec, Morelos, México

Valiyaparambath Musfir Mehaboob Plant Molecular Biology Laboratory, Department of Botany, Jamal Mohamed College, Tiruchirappalli, India

Yesim Yalcin Mendi Faculty of Agriculture, Department of Horticulture, University of Çukurova, Adana, Turkey

Maurizio Micheli Department of Agricultural, Food and Environmental Sciences (DSA3), University of Perugia, Perugia, Italy

Monisha Mitra Department of Agricultural Biotechnology, Faculty of Agriculture, Bidhan Chandra Krishi Viswavidyalaya, Mohanpur, Nadia, West Bengal, India

Eridiana Beatriz Montoya-Medina Natural Products Laboratory, Biotechnology Department, Centro de Desarrollo de Productos Bióticos, Instituto Politécnico Nacional, Yautepec, Morelos, México

Nazatul Asikin Muda Agricutural Biotechnology Research Group, Faculty of Plantation and Agrotechnology, Universiti Teknologi MARA, Shah Alam, Selangor, Malaysia

Débora De Oliveira Prudente Universidade Federal de Lavras, Lavras, Brazil

Canan Yüksel Özmen Biotechnology Institute, Ankara University, Ankara, Turkey

Renato Paiva Universidade Federal de Lavras, Lavras, Brazil

Ruchira Pandey Tissue Culture and Cryopreservation Unit, ICAR-National Bureau of Plant Genetic Resources (NBPGR), Pusa Campus, New Delhi, India

Ranjith Pathirana The New Zealand Institute for Plant and Food Research Limited, Palmerston North, New Zealand

Ahmad A. Qahtan Department of Botany & Microbiology, College of Sciences, King Saud University, Riyadh, Saudi Arabia

Afaque Quraishi School of Studies in Biotechnology, Pt. Ravishankar Shukla University, Raipur, India

Umme Salma Department of Agricultural Biotechnology, Faculty of Agriculture, Bidhan Chandra Krishi Viswavidyalaya, Mohanpur, Nadia, West Bengal, India

Akansha Saxena Gosling Research Institute for Plant Preservation (GRIPP), Department of Plant Agriculture, University of Guelph, Guelph, ON, Canada

Praveen Saxena Gosling Research Institute for Plant Preservation (GRIPP), Department of Plant Agriculture, University of Guelph, Guelph, ON, Canada

Basar Sevindik Vocational High School, Izmir Demokrasi University, İzmir, Turkey

Anwar Shahzad Plant Biotechnology Section, Department of Botany, Aligarh Muslim University, Aligarh, Uttar Pradesh, India

Appakan Shajahan Plant Molecular Biology Laboratory, Department of Botany, Jamal Mohamed College, Tiruchirappalli, India

Neelam Sharma Tissue Culture and Cryopreservation Unit, ICAR-National Bureau of Plant Genetic Resources (NBPGR), Pusa Campus, New Delhi, India

Ashley Shepherd USDA-ARS National Laboratory for Genetic Resources Preservation, Fort Collins, CO, USA

Mukund Shukla Gosling Research Institute for Plant Preservation (GRIPP), Department of Plant Agriculture, University of Guelph, Guelph, ON, Canada

Vikram Singh School of Studies in Life Sciences, Pt. Ravishankar Shukla University, Raipur, India

Chellappan Soundar Raju Department of Botany, Horticulture Division, Vivekananda College, Madurai, India

Lucas Batista De Souza Universidade Federal de Lavras, Lavras, Brazil

Alvaro Standardi Department of Agricultural, Food and Environmental Sciences (DSA3), University of Perugia, Perugia, Italy

Monoj Sutradhar Department of Agricultural Biotechnology, Faculty of Agriculture, Bidhan Chandra Krishi Viswavidyalaya, Mohanpur, Nadia, West Bengal, India

José Luis Trejo-Espino Natural Products Laboratory, Biotechnology Department, Centro de Desarrollo de Productos Bióticos, Instituto Politécnico Nacional, Yautepec, Morelos, México

Gabriela Trejo-Tapia Natural Products Laboratory, Biotechnology Department, Centro de Desarrollo de Productos Bióticos, Instituto Politécnico Nacional, Yautepec, Morelos, México

Mehmet Tutuncu Faculty of Agriculture, Department of Horticulture, Ondokuz Mayıs University, Samsun, Turkey

Gayle Volk USDA-ARS National Laboratory for Genetic Resources Preservation, Fort Collins, CO, USA

Qiao-Chun Wang State Key Laboratory of Crop Stress Biology in Arid Region, College of Horticulture, Northwest A&F University, Yangling, Shaanxi, People's Republic of China

Buhara Yücesan Department of Seed Science and Technology, Faculty of Natural and Agricultural Sciences, Bolu Abant İzzet Baysal University, Bolu, Turkey
Horticultural Science Department, North Carolina State University, Raleigh, NC, USA

An Introduction to Synthetic Seeds: Production, Techniques, and Applications

Ahmad A. Qahtan, Eslam M. Abdel-Salam, Abdulrahman A. Alatar, Qiao-Chun Wang, and Mohammad Faisal

Abstract Recent breakthroughs in in vitro culturing of plant cell tissue have helped to overcome the challenges that face important economic and medicinal plant species. Micropropagation and encapsulation techniques have been combined to develop a new tool, known as "synseed," which has the advantages of both technologies. Synseeds or artificial seeds are alginate encapsulated somatic embryos, vegetative buds, or any other micropropagules that can be used as seeds and germinated into plantlets after propagating under in vitro or in vivo conditions and that can also sustain the regeneration potential after low temperature storage. Encapsulated propagules may be used for germplasm preservation of elite plant species and exchange of plant materials between national and international laboratories. In addition, the technology has been successfully utilized for cryopreservation via encapsulation-dehydration, and encapsulation–vitrification for the germplasm storage of elite plant species. In this paper, we provide updated and comprehensive information on synseed technology, with a particular focus on the importance of explant selection for successful synseed production and on the matrices used as an encapsulation material for synseeds. Furthermore, the limiting factors that hinder the progress of synseed technology and related future perspectives are also discussed.

Keywords Conservation · Elite species · Germplasm · Synseeds · Tissue culture

A. A. Qahtan · E. M. Abdel-Salam · A. A. Alatar · M. Faisal (✉)
Department of Botany and Microbiology, College of Sciences, King Saud University, Riyadh, Saudi Arabia

Q.-C. Wang
State Key Laboratory of Crop Stress Biology in Arid Region, College of Horticulture, Northwest A&F University, Yangling, Shaanxi, People's Republic of China

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1 Introduction

Synthetic seeds, or artificial seeds, are encapsulated plant tissues such as shoot buds, axillary buds, somatic embryos, shoot tips, cell aggregates, or any other tissues that can be cultured as a seed and grown into a complete plant under either in vitro or in ex vitro conditions and have the potential to retain their viability after cold storage (Magray et al. 2017; Rihan et al. 2017). Previously, artificial seeds were produced by encapsulation of the somatic embryo; however, in recent years, synseeds have been produced by the encapsulation of various in vitro-derived propagules such as nodal segments containing axillary buds, apical shoot buds, and stem segments (Bapat et al. 1987; Danso and Ford-Lloyd 2003; Rai et al. 2008a). Murashige (1977) was the first researcher to discuss the concept of artificial seeds, while desiccated artificial carrot seeds were first produced by Kitto and Janick (1982). Later, Redenbaugh et al. (1984) successfully developed a method for synseed production by encapsulation of somatic embryos of alfalfa in sodium alginate. Similarly, Bapat et al. (1987) also succeeded in producing synthetic seeds of *Morus indica* from shoot buds encapsulated in alginate and in agar as an alternative to somatic embryos.

During recent decades, there has been great interest in the use of synseed technology to produce artificial seeds, especially for the plants that have low seed viability, seedless fruit, and poor germination rates, as well as for plants that depend on mycorrhizal–fungal symbiosis for germination (Rai et al. 2008b; Gantait et al. 2015). Furthermore, synseed technology may be useful in the selection of genotypes and sterile unsteady genotypes; germplasm preservation of elite planting materials; and in vitro propagation of endangered, rare, and commercially important plants (Danso and Ford-Lloyd 2003; Naik and Chand 2006; Gantait et al. 2015). Additionally, encapsulation technology provides easy handling, short- and long-term storage capacity, genetic uniformity, and low cost quality plant materials; it also allows for transportation and exchange of germplasm between national and international laboratories (Rai et al. 2009; Parveen and Shahzad 2014). Synseed technology has been successfully applied to numerous plant species, including medicinal plants, ornamentals, vegetables, fruits, cereals, and forest trees (Table 1). Schematic representation of synseed production is depicted in Fig. 1.

2 Selection of Plant Materials

Selection of the most appropriate explant as a starting material is a key factor for the successful production of synseeds. Synseeds have been produced from different plant propagules, which are discussed in this section.

Table 1 Application of synseed technology in different plant species

Plant species	Explant	Concentration of sodium alginate	Concentration of calcium chloride	References
Allium sativum	Callus	1.5%	50 mM	Kim and Park (2002)
Manihot esculenta	Nodal cuttings and shoot tips	3%	100 mM	Danso and Ford-Lloyd (2003)
Paulownia elongata	Somatic embryos	1, 2.5, and 3%	50, 60, 80 mM	Ipekci and Gozukirmizi (2003)
Oryza sativa	Somatic embryos	4%	1.5%	Kumar et al. (2005)
Rotula aquatica	Somatic embryos	3%	50 mM	Chithra et al. (2005)
Pinus patula	Somatic embryos	1.5, 2, 2.5, and 3%	100 mM	Malabadi and Staden (2005)
Rhodiola kirilowii	Axillary buds and callus	4 and 5%	50 mM	Zych et al. (2005)
Arnebia euchroma	Somatic embryos	3%	100 mM	Manjkhola et al. (2005)
Hibiscus moscheutos	Nodal segments	2.75%	50 mM	West et al. (2006)
Punica granatum	Nodal segments	1–6%	50, 75, 100, and 125 mM	Naik and Chand (2006)
Chonemorpha grandiflora	Shoot tips	3%	50 mM	Nishitha et al. (2006)
Tylophora indica	Nodal segments	1–5%	25, 50, 75, 100, and 200 mM	Faisal and Anis (2007)
	Nodal segments	2–5%	75 and 100 mM	Gantait et al. (2017b)
Pogonatherum paniceum	Shoot buds	3%	2%	Wang et al. (2007)
Pinus radiata	Somatic embryos	1, 2, and 3%	50, 75, and 100 mM	Aquea et al. (2008)
Psidium guajava	Shoot tips	2–4%	100 mM	Rai et al. (2008b)
Nothofagus alpina	Somatic embryos	2, 3, and 4	5.5, 14, and 15 g/L^{-1}	Cartes et al. (2009)
Zingiber officinale	Microshoots	4%	100 mM	Sundararaj et al. (2010)
Vitex negundo	Nodal segments	2–5%	25, 50, 75, 100, and 200 mM	Ahmad and Anis (2010)
Eclipta alba	Nodal segments	2–5%	50, 100, and 150 mM	Singh et al. (2010)
Solanum nigrum	Shoot tip	2–4%	100 mM	Verma et al. (2010)

(continued)

Table 1 (continued)

Plant species	Explant	Concentration of sodium alginate	Concentration of calcium chloride	References
Khaya senegalensis	Shoot tips	3%	100 mM	Hung and Trueman (2011)
Salvia officinalis	Shoot tips	2 and 3%	50 mM	Grzegorczyk and Wysokińska (2011)
Corymbia torelliana × *C. citriodora*	Shoot tips and nodal segments	3%	100 mM	Hung and Trueman (2012)
Ruta graveolens	Nodal segments	2–5%	25, 50, 75, 100, and 200 mM	Ahmad et al. (2012)
Rauvolfia tetraphylla	Microshoots	1–5%	25, 50, 75, 100, and 200 mM	Alatar and Faisal (2012)
Clitoria ternatea	Somatic embryos	3, 4, and 5%	75 and 100 mM	Kumar and Thomas (2012)
Rauvolfia serpentina	Nodal segments	3%	100 mM	Faisal et al. (2012)
	Shoot tips	1–5%	75 and 100 mM	Gantait et al. (2017a)
Cymbidium	Protocorm-like bodies	3, 3.5, and 4%	100 mM	da Silva (2012)
Dendrobium nobile	Protocorm-like bodies	3%	100 mM	Mohanty et al. (2013)
Rhinacanthus nasutus	Somatic embryos	4%	100 mM	Cheruvathur et al. (2013)
Withania somnifera	Nodal segments with axillary buds	2–5%	25, 50, 75, 100, and 200 mM	Fatima et al. (2013)
Aristolochia tagala	Microshoots	2, 3, and 4%	68 mM	Remya et al. (2013)
Ceropegia bulbosa	Nodal explants	1–5%	100 mM	Dhir and Shekhawat (2013)
Phyllanthus fraternus	Nodal segments	1, 1.5, 2, 2.5, 3, and 4%	25, 50, 75, 100, and 200 mM	Upadhyay et al. (2014)
Ocimum gratissimum	Microshoots	1–5%	25, 50, 75, 100, and 150 mM	Saha et al. (2014)
Terminalia arjuna	Shoot tips	2–5%	100 mM	Gupta et al. (2014)
Cucumis sativus	Shoot tips	1–5%	25, 50, 75, and 100 mM	Adhikari et al. (2014)

(continued)

Table 1 (continued)

Plant species	Explant	Concentration of sodium alginate	Concentration of calcium chloride	References
Anethum graveolens	Somatic embryos	1–5%	75 and 100 mM	Dhir et al. (2014)
Balanites aegyptiaca	Nodal segments	2–5%	25, 50, 75, 100, and 200 mM	Varshney and Anis (2014)
Sterculia urens	Nodal segments	2, 4, and 6	100 mM	Devi et al. (2014)
Cassia angustifolia	Nodal segments	1–5%	25, 50, 75, 100, and 200 mM	Parveen and Shahzad (2014)
Mondia whitei	Somatic embryos	1–4%	75, 100, and 125 mM	Baskaran et al. (2015)
Vitex trifolia	Nodal segments	1–5%	25, 50, 75, 100, and 200 mM	Ahmed et al. (2015)
	Nodal segments	2–5%	25, 50, 75, 100, and 200 mM	Alatar et al. (2017)
Gossypium hirsutum	Axillary buds	1–5%	25, 50, 75, 100, and 200 mM	Hu et al. (2015)
Ledebouria revoluta	Somatic embryos	1.5, 3, and 4.5%	150 mM Ca $(NO_3)_2$	Haque and Ghosh (2016)
Solanum tuberosum	Axillary buds	2.5, 3, and 3.5%	1 and 1.5%	Ghanbarali et al. (2016)
Curcuma amada	Somatic embryos	1–4%	100 mM	Raju et al. (2016)
Erythrina variegata	Nodal segments	1–4%	25, 50, 75, 100, and 200 mM	Javed et al. (2017)
Urginea altissima	Shoot tips	3%	100 mM	Baskaran et al. (2017)
Spathoglottis plicata	Protocorm-like bodies	1.5, 3, and 4.5%	3% calcium nitrate	Haque and Ghosh (2017)
Capparis decidua	Nodal segments	2–5%	25, 50, 75, 100, and 125 mM	Siddique and Bukhari (2018)
Ceropegia barnesii	Nodes	2, 3, and 4%	60 mM	Ananthan et al. (2018)
Rosa damascena trigintipetala	Axillary buds	2, 4, and 5%	75 and 100 mM	Attia et al. (2018)
Salix tetrasperma	Nodal segments	1–5%	25, 50, 75, 100, and 200 mM	Khan et al. (2018)
Plumbago rosea	Nodal axillary buds	2.5, 3, 4, and 5%	50, 75, 100, and 200 mM	Prakash et al. (2018)

(continued)

Table 1 (continued)

Plant species	Explant	Concentration of sodium alginate	Concentration of calcium chloride	References
Taraxacum pieninicum	Shoot tips	3%	100 mM	Kamińska et al. (2018)
Saccharum officinarum	Microshoots	2, 3, and 4%	25, 50, 75, 100, and 125 mM	Badr-Elden (2018)

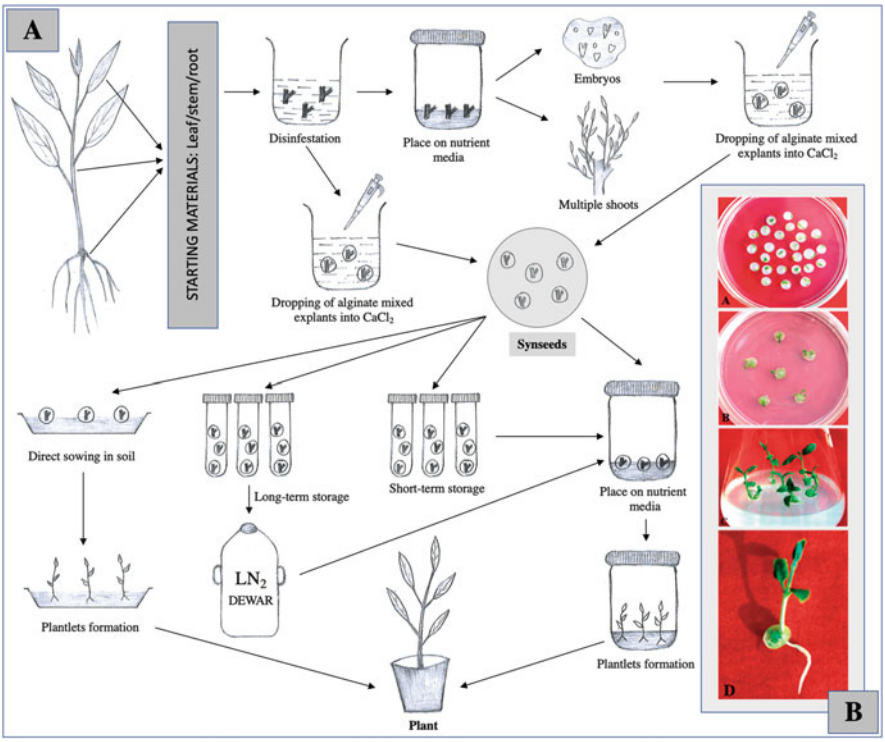

Fig. 1 (**a**) Schematic representation of synthetic seed production; (**b**) synseed seed produced from nodal segments of *Tylophora indica*. Source: Faisal and Anis (2007)

2.1 Somatic Embryo

Bipolar structures that contain both the shoot and root poles are described as somatic embryos. These are the most suitable material for synseed seed production because of their polar nature, which means they are able to develop roots and shoots in a single step (Standardi and Piccioni 1998; Sharma et al. 2013). Somatic embryos have been successfully used for synseed production in several plant species including

Rotula aquatica (Chithra et al. 2005), *Oryza sativa* (Kumar et al. 2005), *Pinus radiata* (Aquea et al. 2008), *Nothofagus alpina* (Cartes et al. 2009), *Dalbergia sissoo* (Singh and Chand 2010), *Clitoria ternatea* (Kumar and Thomas 2012), *Rhinacanthus nasutus*, *Hemidesmus indicus* (Cheruvathur et al. 2013), *Anethum graveolens* (Dhir et al. 2014), *Mondia whitei* (Baskaran et al. 2015), *Ledebouria revoluta* (Haque and Ghosh 2016), and *Curcuma amada* (Raju et al. 2016). However, the deficient and asynchronous maturation of the embryonic pole is the basic problem for synseed production in woody species (Cartes et al. 2009; da Silva and Malabadi 2012). To address this problem, several researchers proposed using compounds such as nutrients, growth regulators, herbicides, anti-pathogens, bio-fertilizers, and bio-controllers (Kumar et al. 2005; Cartes et al. 2009). Somatic embryos of *Pinus patula* that had been encapsulated with 2.5% sodium alginate and dissolved in DCR basal medium had a germination rate of 89% (Malabadi and Staden 2005). Cheruvathur et al. (2013) reported that synseeds produced from somatic embryos had a 100% regeneration rate in MS with 2 μM kinetin and 0.5 μM IBA.

2.2 Nodal Segment and Shoot Tips

Nodal segments with axillary bud (microcuttings) are the most common propagules used for synseed production. This is probably due to the relative ease with which these explants are produced once the micropropagation system has been established and because they have the ability to retain viability in terms of sprouting and conversion potential even after a considerable period of storage, which is required for germplasm exchange (Piccioni and Standardi 1995; Ahmad et al. 2012). Nodal segments have been frequently used for synthetic seeds in several plants, such as *Tylophora indica* (Faisal and Anis 2007; Gantait et al. 2017b), *Eclipta alba* (Singh et al. 2010), *Vitex negundo* (Ahmad and Anis 2010), *Ruta graveolens* (Ahmad et al. 2012), *Rauvolfia serpentina* (Faisal et al. 2012), *Cannabis sativa* (Lata et al. 2012), *Ceropegia bulbosa* (Dhir and Shekhawat 2013), *Sterculia urens* (Devi et al. 2014), *Balanites aegyptiaca* (Varshney and Anis 2014), *Phyllanthus fraternus* (Upadhyay et al. 2014), *Centella asiatica* (Prasad et al. 2014), *Vitex trifolia* (Ahmed et al. 2015; Alatar et al. 2017), *Gossypium hirsutum* (Hu et al. 2015), *Solanum tuberosum* (Ghanbarali et al. 2016), *Erythrina variegata* (Javed et al. 2017), *Capparis decidua* (Siddique and Bukhari 2018), *Salix tetrasperma* (Khan et al. 2018), and *Rosa* × *damascena* f. *trigintipetala* (Attia et al. 2018).

Apical meristems or shoot tips were also used for encapsulation of explants in several plant species, such as *Psidium guajava* (Rai et al. 2008b), *Solanum nigrum* (Verma et al. 2010), *Khaya senegalensis* (Hung and Trueman 2011), *Salvia officinalis* (Grzegorczyk and Wysokińska 2011), *Cucumis sativus* (Adhikari et al. 2014), *Terminalia arjuna* (Gupta et al. 2014), *Rauvolfia serpentina* (Gantait et al. 2017a), *Urginea altissima* (Baskaran et al. 2017), and *Taraxacum pieninicum* (Kamińska et al. 2018). Hung and Trueman (2012) successfully developed methods for synseed production in *Corymbia torelliana* × *C. citriodora* using nodal segments and shoot tips. They found higher regrowth abilities, with about 76–100% regrowth

from nodal segments and 78–100% from encapsulated shoot tips, using full and half-strength MS media, respectively.

2.3 Callus and Protocorm-Like Bodies

Generally, calluses are not often used in production of synseeds. This could be attributed to the undifferentiated nature of calluses, which have several requirements for successful differentiation that limits the utility and acceptability of the use of calluses in the production of synseeds (Gantait et al. 2015). There have been very few successful attempts to produce synseeds by encapsulating calluses. In a previous study, calluses of *Allium sativum* obtained in vitro from shoot tip explants were encapsulated using calcium chloride and sodium alginate and regenerated on semi-solid ½MS medium without growth regulators, and achieved a regeneration frequency of 95% (Kim and Park 2002). Similarly, Zych et al. (2005) successfully encapsulated the differentiating calluses derived from the hypocotyls of *Rhodiola kirilowii* plants. The encapsulated calluses can be stored at a low temperature (4 °C) for 6 weeks and exhibited regeneration potential after transfer without hormones, with 95% regeneration frequency.

The production of synthetic seeds using protocorm-like bodies (PLBs) is mainly used for orchids because they produce tiny, non-endospermic seeds. Several studies have investigated the feasibility of encapsulating PLBs and culturing the produced seeds directly in the soil, without in vitro regeneration (e.g., Ara et al. 2000; Saiprasad 2001; Vij et al. 2001; Saiprasad and Polisetty 2003). Corrie and Tandon (1993) found that encapsulated PLB of *Cymbidium giganteum* could be cultivated directly in sterilized soil with a regeneration frequency of 88 and 64% on sand and on a sand and soil mixture, respectively. Seeking to optimize seed production in three orchid genera (*Dendrobium*, *Oncidium*, and *Cattleya*), Saiprasad and Polisetty (2003) tried different developmental stages of PLBs and various combinations of sodium alginate, $CaCl_2$, and MS salts. They successfully encapsulated fractionated PLBs after 13–15 days of culture using 3% sodium alginate and 75 mM $CaCl_2$. Seeds of *Dendrobium*, *Oncidium*, and *Cattleya* were stored at 4 °C for 75, 60, and 30 days, respectively, and had a regeneration potential of more than 88%. Sarmah et al. (2010) used PLB produced from 6-week-old leaves of *Vanda coerulea* plants for encapsulation using 100 mM $CaCl_2$ solution for 30 min, and the encapsulated PLBs were stored for 100 days at 4 °C.

3 Selection of the Encapsulation Matrix

The encapsulation material is considered to be a critical factor for the production of uniform synseeds. The encapsulation material should be consistent enough to allow seed handling without breakage, but weak enough to allow the bud to break free

from the capsule upon regrowth (Redenbaugh et al. 1986). This balance between synseed hardness and softness can be achieved by encapsulating explants with sodium alginate hydrogel (Rai et al. 2009; Gantait et al. 2015). Sodium alginate is the most commonly used substance for encapsulation of explants; however, there are other agents such as sodium alginate with gelatin, potassium alginate, sodium pectate, and carrageenan that are used for encapsulation. In general, sodium alginate has been shown to be the most commonly used for encapsulation because of its useful thickness, low cost, fast gelation, and nontoxic nature (Rai et al. 2009; Cheruvathur et al. 2013; Gantait et al. 2015; Rihan et al. 2017). It can also provide better protection for the covered explants against mechanical damage (Saiprasad 2001). The strength of encapsulated beads depends mainly on the concentration of sodium alginate and calcium chloride, as well as the mixing duration; however, it may vary for different explants and plant species (Rai et al. 2009; Rihan et al. 2017). Furthermore, the addition of nutrients and growth regulators to the encapsulation matrix is also an important factor for successful synseed production, as it increases the reliability of germination and the viability of the synseeds. These matrices are considered to be artificial endosperms, and they also play an important role in the storage of synseeds at low temperatures and in regrowth ability after transfer to germination media (Saiprasad 2001; Rihan et al. 2017).

In most studies, the optimum concentration for synthetic seed production has been reported to be 3% sodium alginate and 100 mM $CaCl_2$ for several plant species including *Manihot esculenta* (Danso and Ford-Lloyd 2003), *Tylophora indica* (Faisal and Anis 2007), *Psidium guajava* (Rai et al. 2008a, b), *Vitex negundo* (Ahmad and Anis 2010), *Eclipta alba* (Singh et al. 2010), *Solanum nigrum* (Verma et al. 2010), *Khaya senegalensis* (Hung and Trueman 2011), *Ruta graveolens* (Ahmad et al. 2012), *Rauvolfia tetraphylla* (Alatar and Faisal 2012), *Rauvolfia serpentina* (Faisal et al. 2012), *Dendrobium nobile* (Mohanty et al. 2013), *Ceropegia bulbosa* (Dhir and Shekhawat 2013), *Balanites aegyptiaca* (Varshney and Anis 2014), *Cucumis sativus* (Adhikari et al. 2014), *Vitex trifolia* (Ahmed et al. 2015; Alatar et al. 2017), *Mondia whitei* (Baskaran et al. 2015), *Curcuma amada* (Raju et al. 2016), *Erythrina variegata* (Javed et al. 2017), *Salix tetrasperma* (Khan et al. 2018), and *Taraxacum pieninicum* (Kamińska et al. 2018). Nevertheless, an encapsulation matrix of 2% sodium alginate with $CaCl_2$ of 50 mM was found to produce high quality beads in *Artemisia vulgaris* by encapsulating nodal segments (Sujatha and Kumari 2008). Wang et al. (2007) reported that *Pogonatherum paniceum* synseeds produced using 3% sodium alginate, 1% activated carbon, and 2% calcium chloride gave a higher conversion rate (61.58%) than synseeds prepared without activated carbon (44.06%). da Silva (2012) reported that 3.5% sodium alginate was the most appropriate concentration for the encapsulation of PLB in hybrid *Cymbidium*. Furthermore, 3% sodium alginate and 75 mM calcium chloride ($CaCl_2 \cdot 2H_2O$) were found to be the most appropriate combination for synseed production in *Tylophora indica* and *Rauvolfia serpentina* (Gantait et al. 2017a, b). Similarly, Siddique and Bukhari (2018) also found that 3% sodium alginate and 75 mM calcium chloride was the best suited matrix for synseed production in *Capparis decidua*.

4 Methods

4.1 Encapsulation Matrix

The required concentrations of sodium alginate solution (0.5–5.0% w/v) were prepared in liquid nutrient medium or double distilled water to form the gel matrix. Similarly, calcium chloride solutions were prepared at different concentrations (25–200 mM) in double distilled water to form the complexing agent. Both the gel matrix and complexing agent were autoclaved at 121 °C and 1.1 kg cm^{-2} pressure for 15 min.

4.2 Encapsulation

After preparation of gel matrix and complexing agent, selected plant materials (explants) were prepared for encapsulation as follows:

1. The propagules were dipped in 3% sodium alginate solution.
2. The mixture (propagules contained within sodium alginate) was placed into calcium chloride solution (100 mM) and left for 30–40 min to allow the alginate beads to harden, forming calcium alginate around the propagules.
3. Calcium alginate beads were washed with sterile double distilled water two to three times to remove traces of calcium chloride.
4. Synseeds were transferred to sterile filter paper and left for 5 min under the laminar air flow hood to dry.
5. The synseeds were then ready and could be stored at 4, 15, or 24 °C, depending on the intended use.

5 Applications of Synseeds

Synseeds have several applications in different fields of plant biotechnology and the conservation of rare or endangered plant species. These applications include in vitro or ex vitro (direct sowing) propagation of various plant species; short-, medium-, and long-term preservation of germplasm; and transportation and exchange of plant materials.

5.1 Propagation

Encapsulated explants are characterized by regrowth and conversion abilities after encapsulation and storage at low temperatures, when transferred to the germination

media (Micheli et al. 2007). Synseeds could be used for propagation and multiplication of rare and endangered plants, elite genotypes, seedless plants, medicinal plants, genetically engineered (modified) plants, and commercially important plants (Rai et al. 2009; Gantait et al. 2015). Synseeds can be efficiently cultivated in vitro, either on semi-solid culture medium or planting substrate (e.g., perlite, vermicompost, vermiculite, soil, soilrite, sand, or gravel) for conversion into complete plantlets (Sharma et al. 2013). Generally, the regrowth ability of explants encapsulated in calcium alginate beads into complete plantlets on nutrient-rich medium is more effective than on nutrient-deficient substrates (Mandal et al. 2000; Sharma et al. 2013). The concentration of plant growth regulators in the medium plays a crucial role in conversion and whole plant regeneration from encapsulated buds (Cheruvathur et al. 2013). The plant growth regulator requirement in nutrient medium significantly depends upon the plant species. Nishitha et al. (2006) reported that encapsulated shoot tips of *Chonemorpha grandiflora* had a 95% conversion to plantlets on medium containing 0.49 μM IBA and 11.7 μM silver nitrate. Plantlets showed a 90% survival after acclimatization in soil. Dhir and Shekhawat (2013) reported that maximum percentage response for conversion of synthetic seeds into plantlets in *Ceropegia bulbosa* was 100% on medium supplemented with 8.88 μM BA. Gupta et al. (2014) found that highest rate of plantlet conversion from encapsulated shoot tips in *Terminalia arjuna* on 0.14% gelrite-gelled MMS supplemented with 0.5 mg L^{-1} BAP and 0.1 mg L^{-1} NAA was 91.6%. Encapsulated somatic embryos of *Mondia whitei* had 95.7% survival and 73% germination rates (Baskaran et al. 2015). Baskaran et al. (2017) obtained 91% adventitious shoot regeneration from *Urginea altissima* encapsulated shoot tips on semi-solid MS medium containing 10 μM *m*T and 2 μM NAA. Siddique and Bukhari (2018) obtained the highest conversion rate of 93% from encapsulated nodal segment of *Capparis decidua*.

5.2 Short- and Medium-Term Conservation

Synseed technology offers strategies for the conservation of plant species through short- and medium-term preservation. These processes are generally known as slow-growth techniques. Appropriate storage conditions and a finite storage period are the most critical factors to maintain synseed viability during transportation and conservation, and these may lead to the successful commercialization of this technique (Sharma et al. 2013). The optimal storage temperature for short- or medium-term storage varies depending on the plant species. Generally, low temperature storage at 4 °C in a laboratory freezer has been found to be the most suitable conditions for synseeds of most plant species (Ray and Bhattacharya 2008; Parveen and Shahzad 2014; Ahmed et al. 2015; Alatar et al. 2017). The role of temperature on short- or medium-term storage of synseeds has been investigated by several researchers (Table 2). Faisal et al. (2013) reported that 4 °C was the optimal temperature for short-term storage (storage for up to 4 weeks), with a high conversion percentage (80.6%). In *Ceropegia bulbosa*, the conversion frequency of encapsulated nodal

Table 2 Short- and medium-term conservation of synthetic seeds

Plant species	Encapsulated explant	Storage conditions	Storage period	Regrowth rate (%)	Optimum	Post-storage treatment or culture	References
Pinus patula	Somatic embryo	2 °C 4 °C	0–120 days	73–89 61–73	73% at 2 °C after 120 days	½ DCR basal medium	Malabadi and Staden (2005)
Tylophora indica	Nodal segments	4 °C	0–8 weeks	50.3–91.3	72.3 after 4 weeks	MS + 2.5 µM BA + 0.5 µM NAA	Faisal and Anis (2007)
Rauvolfia serpentina	Shoot tips	4 °C	0–14 weeks	68.5–100		MS + 3% sucrose	Ray and Bhattacharya (2008)
	Nodal segments	4 °C	0–8 weeks	50–91.6	80% after 4 weeks	WPM + 5.0 µM BA + 1.0 µM NAA	Faisal et al. (2012)
Zingiber officinale	Microshoots	25 °C	2–12 weeks	13.0–86.7	53 after 8 weeks	MS + 2.5 mg/L BA	Sundararaj et al. (2010)
Vitex negundo	Nodal segments	4 °C	0–8 weeks	50–92.6		MS + 2.5 µM Kin + 1.0 µM NAA	Ahmad and Anis (2010)
Eclipta alba	Nodal segments	4 °C	0–60 days	51.2–100		MS + 0.88 µM BAP	Singh et al. (2010)
Decalepis hamiltonii	Nodal segments	4 °C	0–8 weeks	14–77	63.80 after 2 weeks	MS + 5 µM BA + 0.5 µM IAA + 30 µM ADS	Sharma and Shahzad (2012)
Withania somnifera	Nodal segments with axillary buds	4 °C	2–8 weeks	50.5–86.2	86.2% after 4 weeks	MS + 2.5 µM BA + 0.5 µM NAA	Fatima et al. (2013)
Hemidesmus indicus	Somatic embryos	4 °C	4 months	86–100	90% after 90 days	MS + 2 µM Kn + 0.5 µM IBA	Cheruvathur et al. (2013)
Cassia angustifolia	Nodal segments	4 °C	0–8 weeks	43.90–94.06	72.30% after 4 weeks	MS + 2.5 µM BA + 0.4 µM NAA	Parveen and Shahzad (2014)
Sterculia urens	Nodal segments	4 °C	0–6 months	73.33–95	73.33 after 6 months	MS + 0.2 mg L^{-1} TDZ	Devi et al. (2014)

Ocimum kilimandscharicum	Shoot tip	4 and 25 °C	30–60 days 30–90 days	23.60–9.72 54.16–81.94	81.94% at 25 °C after 30 days	MS + 1.0 mg/L BA	Saha et al. (2015)
Vitex trifolia	Nodal segments	4 °C, 15 °C, and 24 °C	0–8 weeks	42.5–92.3	74.5% after 4 weeks	MS + 5.0 μM BA + 0.5 μM NAA	Ahmed et al. (2015)
	Nodal segments	4 °C	2–12 weeks	48–97	71.6% after 8 weeks	MS + 2.5 μmol/L KN + 1.0 μmol/L NAA	Alatar et al. (2017)
Curcuma amada	Somatic embryos	4 °C and 25 °C	0–120 days	54.16–86.11 37.49–72.22	88.10% at 4 °C after 30 days	½ MS + 0.25 mg L^{-1} GA_3	Raju et al. (2016)
Urginea altissima	Shoot tips	4 and 25 ± 2 °C	15 days	67.6% at 4 °C and 91% at 25 ± 2 °C	91% at 25 ± 2 °C	MS + 10 μM *m*T + 2 μM NAA	Baskaran et al. (2017)
Spathoglottis plicata	Protocorm-like bodies	4 °C, 15 °C, and 24 °C	0–120 days	48–88%, 27–88%, and 18–88%	66.7% after 90 days at 4 °C	MMS + 0.5 mg L^{-1} Kin	Haque and Ghosh (2017)
Capparis decidua	Nodal segments	4 °C	0–6 weeks	73–93	93% after 4 weeks	MS + 5.0 μM TDZ + 0.5 μM IAA	Siddique and Bukhari (2018)
Salix tetrasperma	Nodal segments	4 °C	0–8 weeks	49.67–91.33	71% after 4 weeks	WPM + 2.5 μM Kin + 0.5 μM NAA	Khan et al. (2018)
Rosa damascena trigintipetala	Axillary buds	4 °C	0, 4, and 8 weeks	60–90	60% after 8 weeks	MS + 0.5% sucrose	Attia et al. (2018)

segments was 50.7% after 60 days of storage at 4 °C, while storage of up to 90 days inhibited conversion into plantlets (Dhir and Shekhawat 2013). Fatima et al. (2013) reported that the conversion frequency of *Withania somnifera* synseeds was 86.2% after 4 weeks of cold storage at low temperatures. Devi et al. (2014) observed a 73.33% germination rate from encapsulated nodal segments of *Sterculia urens* plants incubated at 4 °C for 24 weeks. Muthiah et al. (2013) obtained a 86.6% regrowth rate in *Bacopa monnieri* from encapsulated shoot tips and a 60% regrowth rate from encapsulated nodal segments after 6 months of storage at 4 °C. Benelli (2016) observed a 83.3% regrowth rate in encapsulated shoot tips of *Vitis* (*V. berlandieri* × *V. riparia*) "Kober 5BB" grapevine rootstock after 9 months of cold storage (4 °C) in darkness, while the encapsulated nodal segments had a regrowth rate of 55.6% under the same storage conditions. Furthermore, Khan et al. (2018) found that the conversion and development rate of plantlets from encapsulated nodal segments of *Salix tetrasperma* plants was 71% after 4 weeks of storage at 4 °C, while non-encapsulated nodal segments of the same plant had a 30.33% conversion rate for the same time period. The encapsulated somatic embryos of *Curcuma amada* incubated at 4 °C had germination rates of 88.10% after a month of storage and 54.16% after 4 months of storage (Raju et al. 2016). However, Sujatha and Kumari (2008) successfully stored encapsulated nodal segments of *Artemisia vulgaris* for a period of 60 weeks at 5 °C with an 85% survival rate on proliferation medium. Nevertheless, Haque and Ghosh (2016) observed a 57.8% regrowth rate for *Ledebouria revoluta* encapsulated somatic embryos incubated at 15 °C after 4 months of storage, while the synthetic seeds stored at 24 and 4 °C had regrowth rates of 26.7 and 0% after the same storage period, respectively. For *Tylophora indica* synseeds, storage at 15 ± 1 °C for 45 days was more optimal for regrowth than either 5 ± 1 °C or 25 ± 1 °C (Gantait et al. 2017b).

5.3 Transport

Many commercially important plants have been studied for propagation, genetic engineering, breeding, and pharmaceutical purposes. Synseed production technology could be useful for the transportation and exchange of elite germplasms, axenic plant material, and genetically engineered plants between laboratories at national and international levels (Danso and Ford-Lloyd 2003; Naik and Chand 2006; Rai et al. 2008a; Parveen and Shahzad 2014; Rihan et al. 2017). Ahmed et al. (2015) suggested that the high frequency of plantlet retrieval from encapsulated nodal segments of *Vitex trifolia* after 4 weeks of storage at low temperatures could enable this to be used as a delivery system for the exchange of germplasms, and researchers should explore the possibility of using this method for ex situ conservation of this forest plant.

6 Limitations and Future Perspectives

Since synseeds are one of the key techniques that could be utilized for germplasm exchange between countries and for conservation (especially for short- and medium-term conservation), they have attracted the attention of the scientific world in recent years. Nevertheless, the commercialization of this technique cannot be optimized unless several major limitations are resolved. The large-scale production of synseeds with high regeneration ability in a cost-effective manner is the first step that needs to be achieved to allow commercialization of this technique (Ara et al. 2000). Currently, the most suitable plant material for production of synseeds is somatic embryos. However, their utilization as synseeds is limited by asynchronous development, the loss of embryogenic potential with aging cultures, precocious germination, the lack of tolerance to desiccation, and structural anomalies (Mandal et al. 2000; Naik and Chand 2006; Hung and Trueman 2012). Further research is required in this area to solve the problems facing the production of synseeds. Furthermore, additional research is also needed in order to automate the production of synseeds via automation of encapsulation and regeneration methods; this would likely increase the efficiency of production in a cost-effective manner (Pintos et al. 2008). Automated and optimized production of synseeds may aid the utilization of other non-embryogenic propagules for the production of synseeds in plants species that are not able to form somatic embryos. Another limitation for the use of non-embryogenic propagules is the conversion for rooting, especially in woody plants, which requires further research and investigation (Hung and Trueman 2011, 2012). Moreover, optimization and refinement of the existing protocols for production of synseeds of several plant species also requires further research. Germplasm cryopreservation is considered to be one of the main potential applications for synthetic seeds. Therefore, there is a significant need for further research in this area.

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Synthetic Seeds: Relevance to Endangered Germplasm Conservation In Vitro

Akansha Saxena, Mukund Shukla, and Praveen Saxena

Abstract The twentieth century witnessed deterioration of biodiversity and loss of natural habitats of many plant species. However, it was also an era of significant progress in tissue culture technology which opened further vistas for multiplication and conservation of plant species. Synthetic seed technology is one such method which involves selection of a suitable explant and encapsulating it in an apposite matrix for successful germination and conversion into a healthy plantlet. The underlying basis of synthetic seed technology is to imitate natural plant development that occurs through seed germination. This method has been successfully employed for propagation and storage of various forest, medicinal, and vegetable plant species. The technique is of pivotal importance for species which produce non-viable seeds, recalcitrant seeds, or have limited and rare seed production. Threatened and endangered plant species are one such category which has several bottlenecks in seed development, reproduction, and establishment in their natural environments that have undergone disruptive changes. This review aims to explore and assess the potential of synthetic seed technology as an effective approach to support conservation strategies for endangered plant species.

Keywords Synthetic seeds · Endangered plants · Life cycle · Propagation · Conservation

Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
2iP	Isopentenyl adenine
AA	Ascorbic acid
ABA	Abscisic acid

A. Saxena · M. Shukla · P. Saxena (✉)
Gosling Research Institute for Plant Preservation (GRIPP), Department of Plant Agriculture, University of Guelph, Guelph, ON, Canada
e-mail: psaxena@uoguelph.ca

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BAP/BA	6-Benzyladenine
CR	Critically endangered
DD	Data deficient
EC	Embryogenic cells
ES	Endangered species
GA_3	Gibberellic acid
$H_2SO_4^-$	Sulphuric acid
IAA	Indole-3-acetic acid
KN	Kinetin
MS	Murashige and Skoog media
NAA	α-Naphthaleneacetic acid
PGR	Plant growth regulators
SE	Somatic embryo
TDZ	Thidiazuron
tTCL	Thin cell layers

1 Introduction

In the later years of the twentieth century, two unrelated events occurred. In order to protect and conserve nature, and natural resources worldwide, representatives of governments and conservation organisations established the International Union for Conservation of Nature (later renamed as International Union for Conservation of Nature and Natural Resources, IUCN) in 1948. The prime role of this organisation was to impart scientific knowledge, data, and tools to public, private, and non-governmental organisations, to facilitate nature conservation and sustainable development (http://www.iucn.org). In order to assess the global conservation status and extinction risk of biological species, the IUCN Red List of Threatened Species (also known as the IUCN Red List or Red Data List) was prepared in 1964. The criteria used to evaluate biological species included declining or fluctuating population, small population or geographic range size and fragmentation, and quantitative analysis of extinction. The species were divided into several different classes including extinct, extinct in wild, critically endangered, endangered vulnerable, near threatened, least concern, data deficient, and not evaluated (Guidelines for Using the IUCN Red List Categories and Criteria 2017). Apart from classification of plant species into these categories, efforts to save the endangered and threatened plants also began.

The other significant event took place in the year 1977 when Murashige presented the idea of an artificial seed or synthetic seed described as ‘an encapsulated single somatic embryo’ (Murashige 1977). Later in 1985, this theoretical idea was transformed into reality when Kitto and Janick (1985) encapsulated and successfully germinated carrot somatic embryos in polyoxyethylene. With these studies, the development of synthetic seed technique began. In simplest term, the technique

can be described as a method to mimic natural seeds by coating the somatic embryo and meristematic parts of plants in a protective and non-toxic matrix.

These two very unrelated events both address and provide a solution to the problem of plant extinction. The decrease in the number of several plant species due to climate change and human activities poses a severe threat towards extinction of a number of plants, leading to unbalanced ecosystems. However, some plant species have bottlenecks in their life cycle, which further hinder their reproduction and population increase. Some of the approaches towards conservation of endangered plants include in vitro mass multiplication of plants for redistribution in natural or new environments, establishment of seed banks, in situ and ex situ conservation, and cryopreservation of endangered plants. This review aims to explore the question 'can synthetic seed technology facilitate efforts to save endangered plants?'

When synthetic seed production technology was in its infancy, only somatic embryos were used as an explant for encapsulation. However, somatic embryo production is not feasible in all the plant species which has led to some researchers suggesting the use of non-embryogenic tissue as explants. The use of encapsulated shoot buds for propagation in *Morus indica* L. was proposed by Bapat et al. (1987) as a method to overcome lack of somatic embryo induction and difficulty in rooting of the cuttings. The substitution of somatic embryos with non-embryogenic tissues like shoot tips, nodal segments, protocorm-like bodies, and callus further opened new vistas for extension of synthetic seed technology to recalcitrant species. In addition, the use of meristematic tissue can minimise the risk of somaclonal variation (Standardi and Piccioni 1998), which can be of key importance for conserving endangered plant species.

The type of explants to be encapsulated is not restricted only to in vitro produced explants. In some cases, ex vitro produced explants were used for encapsulation. These included vegetative buds from a mature mulberry tree (Pattnaik et al. 1995); in vivo grown microshoots of *Curcuma amada* Roxburgh (Banerjee et al. 2012); axillary buds from garden grown plants of *Ocimum americanum* L. (hoary basil), *O. basilicum* L. (sweet basil), *O. gratissimum* L. (shrubby basil), and *O. sanctum* L. (sacred basil) (Mandal et al. 2000); and axillary buds from greenhouse grown *Chrysanthemum* × *grandiflorum* (Ramat.) Kitam. cv. 'Royal Purple' (Hung and Dung 2015).

Thus, synthetic seeds from ex vitro propagated plant materials could be very useful in broadening the application of this technology in plants for which a micropropagation protocol is not available. Similarly, in the case of endangered plants, where tissue availability for micropropagation protocol development is rather limited, various explants from natural populations can also be used for encapsulation and synthetic seed production.

Synthetic seed production has advantages over natural seed production in certain situations. Natural seeds require dormancy and a drying period. Some seeds are susceptible to seed-borne diseases and insect infestation in field and storage, further adding to the difficulty of processing and conserving them. Additionally, naturally produced seeds are not always true to the type, and some plants do not produce seeds regularly or produce non-viable seeds. In contrast, synthetic seeds are produced

vegetatively and are likely to be true to the type in most cases. They can be produced all around the year and are unaffected by seed-borne diseases and insect damage, as well as are easier to handle and transport. Natural seed production requires more labour, time, and space, while synthetic seed production needs skilled labour, but it is more time and space efficient. Synthetic seed technology has been effectively used for propagation, storage, transportation, and conservation of many forest (Reddy et al. 2012; Gupta and Kreitinger 1993), medicinal (Gantait et al. 2015), and vegetable species (Yussof et al. 2011; Siong et al. 2012). Similarly, synthetic seed technology may be useful for conservation of endangered plants. However, this would require greater understanding of the life cycle of rare and endangered plant species and long-term implications of their use in natural environments.

2 Why Do We Need Synthetic Seeds?

Plant's sexual reproductive life cycle consists of many phases such as seed germination, seedling establishment, plant growth, flowering, fruit development, and seed production. However, in the case of endangered/threatened plants, the cycle is interrupted at one point or another due to disruptions at the physiological, biochemical, and molecular levels, as a result of ecological changes often caused by human activities. Some of the reasons of constraints in seed production in endangered plant species are listed below.

2.1 Seed Dormancy in Rare and Endangered Plant Species

Seed dormancy is a state which allows plant survival during adverse conditions. Several factors such as the type and levels of seed coat phytohormones, light, temperature, soil nutrient status, water potential, and genetics are known to control dormancy and germination (Bentsink and Koornneef 2008; Finch-Savage and Footitt 2017). Seed dormancy has been classified into five classes by Baskin and Baskin (1998)—physiological dormancy, morphological dormancy, morphophysiological dormancy, physical dormancy, and combinational dormancy. Physiological dormancy is the most common type of seed dormancy and is associated with inability of embryo to grow normally due to a physiological limitation (Baskin and Baskin 2004). The physiological dormancy is often released on seed treatment with gibberellic acid and dry storage. Physiological dormancy is found in plants such as critically endangered *Ceropegia odorata* (Srinivasarao et al. 2010), *Echium acanthocarpum* Svent. (Carqué-Álamo et al. 2003), and *Dysophylla yatabeana* Makino (Kwon et al. 2018). Morphological dormancy is characterised by underdeveloped and differentiated embryo which needs time to grow (Baskin and Baskin 2004), and it is found in medicinal plants *Apium graveolens* L. (Jacobsen and Pressman 1979) and *Lonicera caerulea* var. *emphyllocalyx* (Phartyal et al. 2009).

Morphophysiological dormancy in seed is due to underdeveloped embryo and physiological inhibitors of dormancy; it is reported in *Heptacodium miconioides* Rehder, endemic in China (Geneve and Kester 2018), and an endemic Iberian species *Narcissus hispanicus* Gouan (Copete et al. 2011). Combinational dormancy is found in seeds with both physical and physiological dormancy as reported in endemic Western Australian species *Diplopeltis huegelii* Endl. (Turner et al. 2006). Physical dormancy is caused due to the presence of water-impermeable layers of palisade cells in the seed or fruit coat (Baskin et al. 2000). The physical dormancy breaks when seed is able to uptake water or when exposed to heating treatment (Morrison et al. 1998). Physical dormancy has been reported in endangered plant species such as *Iliamna corei* (Sherff) Sherff (Baskin 1997), *Pomaderris walshii* J. C. Millott and K. L. McDougall, *Pomaderris adnata* N. G. Walsh and F. Coates (Natale 2016), and critically endangered *Astragalus nitidiflorus* Jiménez and Pau (Segura et al. 2015) and *Malvella sherardiana* (L.) Jaub and Spach. (Veiga-Barbosa et al. 2016). In normal conditions, seed dormancy breaks when plants are exposed to favourable conditions. However, some plants need treatments such as chilling, dry storage/elevated temperatures, light, leaching, scarification, and exposure to chemicals and fluctuating physio-chemical conditions (Bradbeer 1988) to overcome seed dormancy. Seed dormancy has been reported in the case of several endangered/threatened plants, and attempts have been made in order to overcome this issue. A brief account of approaches to overcome seed dormancy in different plant species is summarised below (Table 1).

2.2 Poor Seed Viability and Seed Germination in Endangered Plants

Seed viability is the capacity of seed to germinate under optimal conditions. A non-viable seed fails to germinate under suitable conditions, even if treated for dormancy removal (Bradbeer 1988). Seed germination can be simply defined as 'emergence of radicle through seed coat' (Copeland and McDonald 2001). The endangered and rare plants lack proper seed viability and germination processes, thus threatening population establishment and reproduction. Plant species such as *Rauwolfia serpentina* (L.) Benth. ex Kurz, an endangered medicinal plant, produces non-viable seeds which need treatment with distillery effluent to promote seed germination (Mishra and Gupta 2017). In *Aquilaria malaccensis* Lam., a critically endangered species (Harvey-Brown 2018), short seed viability is a major problem, and seeds require double layer polynet for germination (Tabin and Shrivastava 2014). In critically endangered (Oates and de Lange 1998) *Chordospartium muritai* Purdie, poor seed germination is due to tough seed coat barrier and requires seed scarification in a range of 20–24 °C (Williams et al. 1996). In some plant species, the environmental factors play a significant role in seed germination. The seeds of endangered cactus *Harrisia portoricensis* Britt. require specific microclimate under the canopies of native shrub species for germination (Rojas-Sandoval and

Table 1 List of plant species with seed dormancy issue and their recovery strategies

No.	Scientific name	Reason for seed dormancy	Recovery method	References
1	*Gentiana lutea* L.	Underdeveloped embryos	Cold stratification at 0 °C	Cuena-Lombraña et al. (2017)
2	*Malvella sherardiana* (L.) Jaub and Spach.	Tough seed coat	Soaking seeds in 96% H_2SO_4 for 3 h	Veiga-Barbosa et al. (2016)
3	*Echium acanthocarpum* Svent.	Physiological dormancy	High temperature (17 °C/20 °C) treatment	Carqué-Álamo et al. (2003)
4	*Silene diclinis* (Lag.) M. Laínz	Germination facilitated by after-ripening period of seed	Storage temperature between 2.7 and 1.6 °C	Mira et al. (2011)
5	*Drosophyllum lusitanicum* (L.) Link	Allelochemical from the soil	Seed treatment for 5 min at 100 °C	Gómez-González et al. (2018)
6	*Dysophylla yatabeana* Makino	Physiological dormancy	Pre-soaking in 1000 mg/L GA_3 and incubation at 30–35 °C	Kwon et al. (2018)
7	*Eryngium maritimum* L.	Underdeveloped embryos	Cold stratification at 5 °C	Necajeva and Ievinsh (2013)
8	*Eryngium viviparum* Gay.	Empty seeds, underdeveloped embryos, and morphophysiological dormancy	1 mg/L GA_3, high incubation temperature	Ayuso et al. (2017)
9	*Euryale ferox* Salisb.	Physiological dormancy	Cold stratification at 4 °C	Imanishi and Imanishi (2014)
10	*Manglietiastrum sinicum* (Y. W. Law) Noot.	Inhibitory substances in seed	Moist chilling at 4 °C and seed treatment at 500 mg/L GA_3	Zheng and Sun (2009)
11	*Malcolmia littorea* (L.) W. T. Aiton	Physiological dormancy	Germination at 10–25 °C	De Vitis et al. (2014)
12	*Podophyllum hexandrum* Royle	Hypocotyl dormancy	Treatment with 200 ppm GA_3 of cotyledonary leaves of 1-week-old seedlings	Kharkwal et al. (2008)
13	*Pomaderris walshii*, *P. adnata*, and *P. vacciniifolia* Reissek.	Physical dormancy	Heat shock treatment at (100 °C)	Natale (2016), Patykowski et al. (2016)
14	*Trapella sinensis* Oliver	Physiological dormancy	Cold stratification	Kato and Kadono (2011)

Meléndez-Ackerman 2012). In critically endangered *Widdringtonia whytei* Rendle (Farjon 2013), seeds require incubation at 20 °C for germination (Chanyenga et al. 2012), and seeds of vulnerable species (http://www.natureserve.org/explorer) *Scirpus ancistrochaetus* Schuyler require stratification at 3–8 °C for 8–12 weeks (Lentz and Johnson 1998).

2.3 Inefficient Pollen and Seed Dispersal in Endangered Plants

Seed dispersal is defined as 'departure of a diaspore (e.g. seed and fruit) from the parent plant' (Howe and Smallwood 1982). The process of seed dispersal prevents competition and predation and promotes establishment of the offspring in new habitats, for seed germination and population regeneration (Liu et al. 2014). In a study conducted by Neuschulz et al. (2016), several factors such as pollination, seed dispersal, seed predation, recruitment, and herbivory were assessed in respect of forest disturbances caused by humans. It was determined that out of all the phases involved in regeneration of plant populations, pollination and seed dispersal, which often require interaction with animals, were negatively affected. In the case of endangered plants, lack of seed dispersal and pollination may also be the limiting factors for plant reproduction and species survival. Lack of effective pollinators is reported for endangered species such as *Erysimum capitatum* ssp. *angustatum* L. and *Oenothera deltoides* ssp. *howellii* L. (Pavlik et al. 1993). In some cases, plant multiplication may remain suppressed due to multiple factors functioning in combination to affect seed and pollen dispersal (Table 2).

2.4 Bottlenecks in Life Cycle of Plants Leading to Poor Seed Production

In the case of some plant species, more than one factor is responsible for limited seed production. In *Rauwolfia serpentina* (L) Benth. ex Kurz., poor seed production and declining plant populations are due to low seed viability, poor seed germination rate, low vegetative propagation rate, over-exploitation, and loss of habitat (Dey and De 2011). In some species, mutualism plays an important factor in seed establishment. In *Sarracenia rubra* ssp. *alabamensis*, an endemic to central Alabama, the lack of sphagnum moss affects seedling recruitment (Chesser and Brewer 2011). In near threatened *Pouteria splendens* (A.DC.) Kuntze the seed survival is impaired due to lack of leaf litter (Sotes et al. 2018).

Table 2 List of plant species with inadequate pollen and seed dispersal

Sr. no.	Scientific name	Cause of poor seed and pollen dispersal	References
1	*Bretschneidera sinensis* Hemsl.	Protogynous, high pollen/ovule ratio, limited numbers of flowering individuals, poor pollen transfer efficacy, weak fruit retention, and short flowering season	Qiao et al. (2012)
2	*Cordylanthus maritimus* ssp. *maritimus* L.	Lack of effective pollinators, canopy disturbances	Parsons and Zedler (1997)
3	*Penthorum chinense* L.	Water-mediated seed dispersal hindered by surface-active agents/detergents in river	Ikeda and Itoh (2001)
4	*Pomaderris vacciniifolia* Reissek	Seed dispersal suppressed by habitat fragmentation	Patykowski et al. (2016)
5	*Anthemis chrysantha* J. Gay	Ombrohydrochory (rain-operated seed dispersal)	Aguado et al. (2012)
6	*Euphorbia brevitorta* P.R.O. Bally	Insect-mediated pollen dispersal is compulsory for fruit set and seed dispersal by harvester ants	Martins (2010)

3 Synthetic Seeds: Techniques and Applications for Endangered Plants

Synthetic seed production draws parallels with natural seed development. The technique involves encapsulation of competent explants in an apposite matrix. The artificial encapsulation matrix is analogous to endosperm, which supplies nutrients and protection to the embryo enclosed within the seed. The method aims to achieve encapsulation of somatic embryos or meristematic explants, in a bead providing ease of handling, transport, storage, and a high conversion percentage. The technique has several components such as selection of explant, encapsulation in a gelling agent, polymerisation in different polymers and the duration of polymerisation, evaluation of germination, and conversion of synthetic seeds to plants.

3.1 Selection of Explants

A suitable, totipotent, and competent explant is the elemental unit of synthetic seed technology. Any part of plant can be used as an explant to initiate culture (Smith 2012). Based on the nature and polarity of explant, it can be categorised as bipolar, unipolar, or callus:

(A) Bipolar Explants: The Explants That Have Two Developmental Axes (Root and Shoot)

1. *Somatic Embryos*

   Somatic embryos originate from somatic cells (which are not usually involved in embryo formation) and develop into a whole plant. The somatic embryo has both a shoot and a root axis and a closed vascular system. The somatic embryo also undergoes maturation and accumulation of storage lipids, carbohydrate, proteins, polyamines, and plant hormones like their zygotic counterparts (Winkelmann 2016). Somatic embryos have been considered the most suitable explant for encapsulation and mass production of synthetic seeds (Redenbaugh 1990). Somatic embryos of the endangered plant species like *Swertia chirayita* (Roxb. ex Fleming. H. Karst.) (Kumar and Chandra 2014) and *Gentiana lutea* L. (Holobiuc and Catana 2012) have been successfully encapsulated and regenerated. Somatic embryogenesis is reported in many endangered plants. Thus, these species can potentially be propagated and conserved through synthetic seed production as briefly summarised in Table 3.
2. *Protocorm-Like Bodies*

   Post germination in orchids, the embryo develops in a unique structure known as a protocorm. The prime function of this reproductive structure is to establish a symbiotic relationship with fungus and formation of shoot apical meristem (Yeung 2017). The protocorms are produced by seeds and protocorm-like bodies (PLBs) are formed from explants primarily in in vitro conditions. The PLBs are bipolar in nature and generate root and shoot upon development (Antony et al. 2011; Gnasekaran et al. 2016). Synthetic seed production by encapsulation of PLB in endangered orchid species *Dendrobium nobile* Lindl. (Mohanty et al. 2013) and in *Vanda coerulea* Griff. ex. Lindl. (Sarmah et al. 2010) has been reported. Synthetic seed production in orchids has an advantage over normal plantlet production as encapsulation of single protocorm would minimise the seedling detachment and sorting (Gantait et al. 2015).

(B) Unipolar Explants

Explants such as nodal segments, shoot tips, and root segments which have one developmental axis are unipolar in nature. All plant species do not have an established somatic embryogenesis protocol; therefore, encapsulation of unipolar explants provides an option. In some plant species, micropropagated explants were encapsulated like in *Salvia splendens* F. Sellow ex R. and S. nodal segments (Sharma et al. 2014a, b), M.26 apple rootstock microcuttings (Brischia et al. 2002), and *Chonemorpha grandiflora* L. shoot tips (Nishitha et al. 2006) to develop artificial seeds. Similarly, various tissues from micropropagated plants of endangered and rare plants can help in production of shoots, roots, and callus which can be encapsulated and used for production of synthetic seed. Micropropagation protocols have been developed for several rare and endangered plants; thus, a vast potential of synthetic seed production exists. The works undertaken on micropropagation of endangered plants under the category of critically endangered and endangered are summarised below (Table 4).

Table 3 List of threatened/endangered plants with established somatic embryogenesis protocols

Sr. no.	Scientific name	Family	Value/use	Conservation status	Explant	Growth regulators for induction of S.E.	References
1	*Acanthopanax seoulenses* Nakai	Araliaceae	Anti-aging properties	Endangered	Seeds	MS + 3.0% sucrose + 0.1–0.2 mg/L ABA	Kang et al. (2014)
2	*Alyssum borzaeanum* Nyár	Brassicaceae	Phytoremediation	DD	Seedling	4.52 μM 2,4-D and 0.92 μM KN	Paunescu (2009)
3	*Angelica glauca* Edgew	Apiaceae	Stomach ailments, rheumatism, urinary disorders, and gynaecological disorders	Endangered	Epicotyl	2.0 μM BA + 2.0 μM NAA	Pandey et al. (2011)
4	*Anoectochilus elatus* Lindley	Orchidaceae	Ornamental chest and abdominal pains and snake bites	Endangered	Nodes/internodes	4.54 μM TDZ + 2.69 μM NAA	Sherif et al. (2018)
5	*Arum palaestinum* Boiss.	Araceae	Anti-cancer and anti-diabetic	Endangered	Corm bud	4.5 μM 2,4-D + 0.46 μM KN, 5.4 μM NAA and 1.7 mM proline	Shibli et al. (2012)
6	*Byrsonima intermedia* A. Juss.	Malpighiaceae	Diarrhea and dysentery	Endangered	Leaf segments/ callus	0.54 μM NAA and 4.76 μM KN	Da Silva et al. (2018)
7	*Calotropis procera* (Aiton) W.T. Aiton	Apocynaceae	Fever, rheumatism, and indigestion	Endangered	Leaf	2.0 mg L^{-1} BAP + 1.0 mg L^{-1} NAA	Sundaram et al. (2011)
8	*Castanea dentata* (Marsh.) Borkh.	Fagaceae	Edible nuts/wood	Apparently secure	Ovule/embryo	0.25 mg/L BA and 6.0 mg/L NAA or 4 mg/L 2,4-D	Merkle et al. (1991)

9	*Chlorophytum borivilianum* Sant. et Fernand	Liliaceae	Anti-tumour activity, impotency, antipyretic	Critically endangered	Seeds	1.16 µM KN + 1.13 µM 2,4-D	Rizvi et al. (2010)
10	*Curculigo orchioides* (Gaertn.)	Hypoxidaceae	Anticarcinogenic	Lower risk near threatened	Rhizome/EC	1.0–4.0 mg/L BAP	Nagesh et al. (2010), Joy et al. (2004)
11	*Eleutherococcus senticosus* Harms.	Araliaceae	Anti-stress	Endangered	Hypocotyl	MS + 4.5 µM 2,4-D	Choi et al. (1999)
12	*Gentiana lutea* L.	Gentianaceae	Gastroprotective	Least concern	Hypocotyl, roots	2.0 mg/L 2,4,5-T	Niiho et al. (2006), Holobiuc and Catana (2012)
13	*Hedychium coronarium* J. Koenig	Zingiberaceae	Anti-diabetic	Endangered	Rhizome/EC	0.5 mg/L BAP	Verma and Bansal (2012)
14	*Iphiona mucronata* L.	Asteraceae	ND	Least concern	Seedling	MS + 0.1 mg/L NAA, 0.1 mg/L KN, 5.0 mg/L AA	Al-Gendy et al. (2013)
15	*Kelussia odoratissima* Mozaff.	Apiaceae	Anti-inflammatory, anti-cancer, neuroprotective	Endangered	Cotyledonary leaves	MS + 1.0 mg/L 2,4-D + 0.25 mg/L KN	Ebrahimi et al. (2018)
16	*Leptadenia pyrotechnica* (Forsk.) Decne	Asclepiadaceae	Rheumatism, asthma Forage crop	Endangered	Callus	4.44 µM BAP and 2.85 µM IAA	Sadeq et al. (2014)
17	*Lilium ledebourii* (Baker) Boiss.	Liliaceae	Ornamental	Vulnerable	Bulblet microscale/ thin cell layer (tTCLs) of young bulblet roots	0.54 µM NAA + 0.44 µM BA	Hosseini (2014), Bakhshaie et al. (2010)
18	*Malaxis densiflora* (A. Rich.) Kuntze	Orchidaceae	Ornamental, cough and hepatic disorders	ND	Protocorm	3.39 µM 2,4-D + 6.80 Mm TDZ	Mahendran and Bai (2016)

(continued)

Table 3 (continued)

Sr. no.	Scientific name	Family	Value/use	Conservation status	Explant	Growth regulators for induction of S.E.	References
19	*Metabriggsia ovalifolia* W. T. Wan	Gesneriaceae	ND	Endemic in China	Leaf	25 µM TDZ	Ouyang et al. (2016)
20	*Oplopanax elatus* L.	Araliaceae	Adaptogenic, anti-convulsant, anti-diabetic	Endangered	Zygotic embryos	0.1 mg/L ABA	Khrolenko and Burundukova (2013), Moon et al. (2006)
21	*Primulina tabacum* Hance	Gesneriaceae	ND	First Class Protected Key Wild Plants of China	Leaf	5.0 µM TDZ	Liang et al. (2010), Ma et al. (2010), Peng and Cheng (2002)
22	*Psoralea corylifolia* L.	Fabaceae	Laxative, aphrodisiac	Endangered	Nodal segments	16 µM TDZ	Faisal et al. (2008)
23	*Musa* spp. cv. Rajeli (AAB)	Musaceae	Snacks/edible	Endangered	Male flower buds	4.0 mg/L 2,4-D, 1.0 mg/L each of IAA and NAA, D-Biotin	Kulkarni and Bapat (2013)
24	*Tapiscia sinensis Oliv.*	Staphyleaceae	Ornamental and afforestation	Vulnerable	Cotyledonary-stage embryos	0.05 mg/L NAA and 0.2 mg/L BA	Wang et al. (2014)
25	*Torreya taxifolia Arm*	Cephalotaxaceae	Furniture and fuel	Critically endangered	Zygotic embryos	0.5 mM 2,4-D, 0.2 mM BA, 0.2 mM KN, 0.1 µM brassinolide, 3.8 µM ABA, 20.5 µM biotin, 1.13 µM folic acid	Ma et al. (2012)
26	*Wedelia calendulacea* Less.	Asteraceae	Anti-inflammatory and antibacterial	Endangered	Leaf	0.5 mg/L 2,4-D	Sharmin et al. (2014)

Gymnosperm							
1	*Tylophora indica* (Burm.f.) Merr.	*Asclepiadaceae*	Asthma, dysentery	Native to Singapore (CR)	Nodal segment	5.0 Mm BA	Sahai et al. (2010)
2	*Ceratozamia mexicana* var. *robusta (Miq.)* Dyer	Zamiaceae	ND	Endangered	Leaves	1.0 mg/L KN + 1.0 mg/L 2,4-D	Chavez et al. (1992)
3	*Pinus armandii* Franch. var. *amamiana* (Koidz.) Hatusima	Pinaceae	Forestry plantation/ lumber	Least concern	Megagametophytes	3.0 μM 2,4-D and 1.0 μM BA	Maruyama et al. (2007)
4	*Encephalartos cycadifolius* (Jacq.) Lehm.	Zamiaceae	ND	Least concern	Callus	1.0 mg/L KN + 1.0 mg/L 2,4-D	Jäger and van Staden (1996)

ND not determined

Table 4 List of critically endangered and endangered plants with established micropropagation protocol

Sr. no.	Scientific name	Family	Value/use	Explant	Growth regulators	Regenerated organs can be encapsulated	References
Conservation status: critically endangered							
1	*Ceropegia Evansii* McCann	Apocynaceae	Antipyretic, analgesic, edible tubers and ornamental flowers	Nodal explants	4.0 mg/L BA and 0.3 mg/L IAA	Shoots	Chavan et al. (2015)
2	*Commiphora wightii* (Arnold) Bhandari	Burseraceae	Anti-inflammatory, anti-bacterial, antimicrobial, and antioxidant	Cotyledonary node/nodal segments	2.68 μM NAA and 4.44 μM BAP	Microshoots/ shoots	Kant et al. (2010), Mohan et al. (2017)
3	*Eugenia singampattiana* Beddome	Myrtaceae	Anti-inflammatory	Nodal segments	1.0 mg/L BA and TDZ	Shoots	Pavendan et al. (2011)
4	*Gentiana kurroo* Royle	Gentianaceae	Expectorant, antibilious, astringent, blood purifier and carminative	Petiole	1.0 mg/L BA, 3.00 mg/L NAA and 0.10 mg/L NAA and 1.0 mg/L TDZ	Callus and shoots	Sharma et al. (2014a, b)
5	*Ilex khasiana* Purakaystha	Aquifoliaceae	ND	Nodal explants/leaf disc	8.88 mM BA 4.64 mM KN/9.04 mM 2,4-D and 2.32 mM BA	Shoots and callus	Dang et al. (2011)
6	*Isoplexis canariensis* (L.) Loud., *I. chalcantha* Svent. and O'Shan., and *I. isabelliana* (Webb and Berth.) Masf.	Plantaginaceae	Cardiac glycosides	Shoot tips and nodal segments	0.5–5 μM BA	Shoots	Pérez-Bermúdez et al. (2002)
7	*Medusagyne oppositifolia* Baker	Ochnaceae	ND	Shoots/nodal segments	3.0 μM BA	Shoots	Marriott and Sarasan (2010)
8	*Sarracenia oreophila* (Kearney) Wherry	Sarraceniaceae	ND	Shoots	9.1 μM trans-zeatin	Shoots	Northcutt et al. (2012)

9	*Thermopsis turcica* Kit Tan, Vural et Küçüködük	Fabaceae	ND	Root and cotyledon	0.5–20 μM NAA	Callus and shoots	Cenkci et al. (2008)
10	*Utleria salicifolia* (Retz.) Wight.	Apocynaceae	Hepatoprotective	Leaf, nodes, and cotyledon	1.0 mg/L BA, 0.1 mg/L KN/0.1 mg/L IAA, 1.0 mg/L BA	Callus shoots	Saradha et al. (2018)
Conservation status: endangered							
1	*Aconitum heterophyllum* Wall	Ranunculaceae	Antipyretic, anti-bacterial	Shoot tips, nodal segments	0.25 mg/L KN and 0.25 mg/L IAA	Shoots	Belwal et al. (2016)
2	*Androcalva perlaria* C. F. Wilkins	Malvaceae	ND	Shoots	1.25 μM KN, 0.125 μM BAP	Shoots	Whiteley et al. (2016)
3	*Ceropegia noorjahaniae* Ans.	Apocynaceae	ND	Nodal explants	2.0 mg/L BAP	Shoots	Chavan et al. (2014)
4	*Cypripedium formosanum* Hayata	Orchidaceae	ND	Axillary buds	44.4 Mm BA	Shoots	Lee (2010)
5	*Gomortega keule* (Molina) Baillon	Gomortegaceae	Ornamental, timber, edible fruits	Zygotic embryo	0.1 mg/L NAA 1.0 mg/L BA	Shoots	Muñoz-Concha and Davey (2011)
6	*Pimelea spicata R.Br.*	Thymelaeaceae	ND	Shoots	MS (no hormones)	Shoots	Offord and Tyler (2009)
7	*Tuberaria major* (Willk.) P. Silva and Rozeira	Cistaceae	ND	Nodal segments	0.2 mg/L BA	Shoots	Gonçalves et al. (2010)
8	*Plantago algarbiensis* Samp.	Plantaginaceae	Aluminium hyper-accumulation	Shoots	BA 0.2 mg dm^{-3}	Shoots	Gonçalves et al. (2009)

ND not determined

1. *Nodal Segments or Microcuttings*

   Nodal segments or microcuttings are shoot segments with one or more buds. Compared to somatic embryos, nodal segments are easier to produce (Piccioni and Standardi 1995), with negligible or limited physiological variation, and easier to store and transport (Benelli 2016; Micheli et al. 2007). Nodal segments have been successfully encapsulated in rare plants such as *Tylophora indica* (Burm. Fil.) Merrill. (Faisal and Anis 2007).
2. *Shoot Tips*

   The use of shoot tips with zones of active meristematic growth is space and cost efficient in synthetic seed production (Fig. 2a, b). Moreover, the encapsulated shoot tips provide ease of transport of propagules in limited space. Shoot tips have been used to develop synthetic seed in various rare plant species like *Mentha arvensis* L. (Islam and Bari 2012) and *Mimosa pudica* L. (Banu et al. 2014).
3. *Hairy Roots*

   The encapsulation of root segments is uncommon for synthetic seed production as compared to use of shoot tips, somatic embryos, and nodal segments. Hairy root segments are developed by genetic modification with *Agrobacterium rhizogenes* Ri (root inducing) plasmids as vectors (Uozumi and Kobayashi 1995). The hairy roots of endangered medicinal plant species like horseradish (Repunte et al. 1996), *Picrorhiza kurrooa Royle* ex Benth. (Rawat et al. 2013), and *Centaurium erythraea* Rafn. (Piątczak and Wysokińska 2013) have been encapsulated successfully. Hairy roots are source of secondary metabolite, and production of synthetic seed can open new avenues for commercial production of valuable chemicals produced by rare medicinal plants.
4. *Microtubers*

   In vitro produced tubers are known as microtubers (Wattimena 1984). Microtubers have been encapsulated and used as synthetic seed in various plants (Ma et al. 2011). Encapsulated microtubers of virus-free seedlings of *Pseudostellaria heterophylla* Rupr. and Maxim. were developed as synthetic seeds, and microtubers produced by several endangered plants like *Gloriosa superba* L. (Yadav et al. 2012), *Ceropegia spiralis* Wight (Murthy et al. 2012), and wild *Cyclamen persicum* Mill. (Karam and Al-Majathoub 2000) have also been encapsulated.

(C) Callus

In synthetic seed production, usually the explants employed are differentiated and preprogramed at the cellular level to develop into plantlets. The undifferentiated callus is however an exception to this trend. In a few cases, callus has been used to develop synthetic seed. The calli of *Rhodiola kirilowii* Rgl. ex Maxim (Zych et al. 2005) and *Allium sativum* L. (Kim and Park 2002) were encapsulated and successfully regenerated. The use of callus may be beneficial for conservation of the plant species for which a micropropagation protocol is unavailable currently, but may be developed in the future.

3.2 Encapsulating Agent

The encapsulating agent is an artificial endosperm which surrounds and protects the explants and also serves as a source of nutrients. The encapsulating agents are technically 'hydrogels' made of hydrophilic polymers which can hold a large amount of water, while maintaining the structure, due to cross-linking of individual polymer chains (Ahmed 2015). The hydrogels mimic natural tissue because of their high-water absorption capacity, porosity, soft consistency, and flexibility. Hydrogels can be prepared with natural polymers like proteins, collagen, gelatine, and polysaccharides such as starch, alginate, and agarose. Agar is extracted from marine algae *Gracilaria* and *Gelidium*. It is heated at 100 °C and cooled and the cells/tissues are embedded in it before it solidifies. Agarose is a neutral gelling agent obtained from agar and used similarly as agar. Carrageenans are extracted from red seaweed such as *Chondrus*, *Eucheuma*, and *Gigartina*. Carrageenan is classified into various types such as lambda (λ), kappa (κ), and iota (ι) based upon percentage of sulphate group attached. The hydrogel commonly used for encapsulation is κ-carrageenans. It is a product of 1,3-linked α-galactose-4-sulphate and 1,4-linked 3,6-β-anhydro galactose. The gelation of κ-carrageenans is induced by alkali metal ions (K^+, Rb^+, Cs^+), alkaline earth metal ions (Ca^+), or trivalent ions (Al^{3+}) and amines. Chitosan is obtained by partial deacetylation of insoluble naturally available chitin, obtained from exoskeletons of crustaceans (Martínez-Ruvalcaba et al. 2007). Vorlop and Klein (1981) encapsulated *E. coli* cells in beads of chitosan-acetate solution dropped in 2% $K_4Fe(CN)_6$ (pH 5.7). Gellan gum is synthesised by bacteria *Pseudomonas elodea* and is similar in characteristics to κ-carrageenans. The encapsulation of gellan gum is promoted in $CaCl_2$ or KCl (Norton and Lacroix 1990). The most commonly used encapsulating agent is sodium alginate due to its non-toxicity, low spin ability, and moderate viscosity which makes it suitable to coat the propagules (Redenbaugh et al. 1988). There are however disadvantages of sodium alginate as it forms a sticky seed coat and allows rapid dehydration of artificial seeds. Advantages and disadvantages of various encapsulating agents are listed in Table 5.

3.3 Polymerising Agent and Polymerising Time Span

The encapsulated explants require an intact shape and structure, along with tolerance to desiccation and mechanical injury, in order to survive in ex vitro conditions and germinate. Ideally, the hydrogel encapsulated explants dropped in polymerising agent should result in beads with optimum rigidity, roundness, and firmness. When sodium alginate encapsulated explant is dropped in calcium chloride (CC) ($CaCl_2 \cdot 2H_2O$), ion exchange between sodium and calcium ions takes place resulting in a bead of calcium alginate. Apart from calcium chloride, calcium nitrate (Inpuay and Te-chato 2012) and potassium nitrate (Onishi et al. 1994) have been used as polymerising agent. Two major factors contribute towards the formation of alginate bead of the required

Table 5 Advantages and disadvantages of common hydrogels used for synthetic seed production[a]

Sr. no.	Hydrogel	Monomer	Advantage	Disadvantage
	Polysaccharides			
1	Agar	1-Agarobiose, 1,3-linked β-D-galactopyranose, and 1,4-linked 3,6-anhydro-α-L-galactopyranose	Good viability of cells, solidified gel can be cut easily	Heat damage to cells
2.	Agarose	Agarobiose	Lower gelling temperature, high purity	NA
3.	κ-Carrageenans	1,3-Linked α-galactose-4-sulphate and 1,4-linked 3,6-β-anhydro galactose	Thermally reversible gel	1. Instability in the presence of ions 2. High gelling temperature
4.	Chitosan	(1–4)2-Amino-β-D-glucose	Antimicrobial	Low viability of plant cells
5	Gellan gum	D-Glucose, L-rhamnose, and D-glucuronic acid	Gels faster and clearer than agar (Kang et al. 1982)	NA
6	Alginate	Alginic acid is a linear (1–4)-linked copolymer of β-D-mannuronate (M) and its C-5 epimer α-L-guluronate (G)	High mechanical strength, high porosity, stability towards monovalent cation	1. Autoclaving reduces polymer viscosity. UV sterilisation preferred (Sakamoto et al. 1995)
	Organic gels			
1	Polyacrylamide	Acrylamide and *N*, *N*′-methylenebisacrylamide	Allows swelling and deswelling of gel beads	Toxic effect of monomers on cells
	Protein gels			
2	Gelatin	Glycine, proline, hydroxyproline	First gelling agent	Stable over a narrow temperature range
	Resin			
3	Polyox	Polyethylene oxide	No growth of microbes, non-toxic imparts desiccation tolerance	NA

NA not available
[a]Suprasanna et al. (2006), Kang et al. (1982)

consistency and shape. First is the concentration of ions in encapsulating agent and polymerising agent to establish ionic bonds. The second factor is polymerisation time which depends on desirable texture of bead and dose of complexing and encapsulating agents. A low concentration of sodium alginate and calcium chloride might lead to formation of fragile beads without definite shape, and a very high concentration might

give rise to rigid and unbreakable beads within a polymerisation time period of 30 min (Taha et al. 2008; Hegde et al. 2017). The complexing time also effects bead to plant conversion. Sarmah et al. (2010) evaluated the germination percentage of encapsulated PLBs of *Vanda coerulea* Grifft. ex. Lindl. in relation to different exposure time to $CaCl_2 \cdot 2H_2O$ solution and determined that best time period was 30 min and the response was lower with shorter and longer duration. A similar effect of 100 mM of $CaCl_2 \cdot 2H_2O$ was reported for germination of synthetic seeds of *Saintpaulia ionantha* Wendl. (Daud et al. 2008). Optimisation of polymerisation time is also important in order to avoid ion toxicity. Nagesh et al. (2009) studied the effect of $CaCl_2$ polymerisation time on shoot development from synthetic seeds of *Curculigo orchioides* Gaertn. The beads produced from 2.5% sodium alginate when exposed to 100 mM $CaCl_2$ for a duration longer than 5 min adsorbed a higher amount of calcium chloride leading to calcium ion toxicity and reduced regeneration of synthetic seed.

3.4 Rinsing

The synthetic seed beads sometimes have sodium and chloride ion residuals attached to their surface. The beads have to be washed in distilled water repeatedly to remove excess ions and limit ion toxicity (Gantait and Kundu 2017). The washed beads are transferred to blotting paper for drying. The dried synthetic seed can be stored, transported, or transferred to regrowth media.

3.5 Evaluation of Germination and Conversion of Synthetic Seeds

Synthetic seed germination is marked by development of the encapsulated embryo into a seedling with cotyledons and roots. The term conversion refers to the development of an embryo into a seedling with true leaves and roots (Redenbaugh 1993). Lai et al. (1995) has described 'germination' in somatic embryos as radicle emergence and 'conversion' as the presence of at least one leaf. Normal seed germination is controlled by environmental factors like ambient temperature, soil moisture level, soil oxygen level, and availability of light/darkness. Apart from all these factors, internal factors such as plant growth regulators (PGRs), especially gibberellin, and nutrient reserves (stored in cotyledons and endosperm) aid in seed germination. The synthetic seed however lacks cotyledons and endosperm; therefore, they have to be transferred on regrowth media to aid germination and enable successful conversion. Regrowth media are enriched with basal salts of commonly used growth media including Murashige and Skoog (MS) (Latif et al. 2007; Pandey and Chand 2005), White's basal medium (Soneji et al. 2002), and Woody Plant Medium (WPM) (Faisal et al. 2013; Nower 2014) as sources of macro- and

micronutrients. The addition of phytohormones improves germination and root development in synthetic seeds (Gantait et al. 2017). Maqsood et al. (2012) tested α-naphthaleneacetic acid (NAA), 6-benzylaminopurine (BAP), and gibberellic acid (GA_3) at various concentrations in germination medium of encapsulated embryos of *Catharanthus roseus* (L.) G. Don. and determined that the best medium was MS + 1.34 μM NAA + 1.10 μM BA. Siddique and Bukhari (2018) evaluated thidiazuron (TDZ) and indole-3-acetic acid (IAA), singly and in combination, and obtained optimum conversion of synthetic seeds of *Capparis decidua* (Forsk.) with MS + 5.0 μM TDZ + 0.5 μM IAA. Encapsulation procedure also affects conversion efficiency of synthetic seeds. For example, Micheli et al. (2002) found that *Malus pumila* Mill synthetic seed conversion was best with encapsulation coating procedure which involves a coating of 2.5% sodium alginate + 2% propylene glycol + 5 g egg albumin over the calcium-alginate bead as compared to simple and double alginate coating.

4 Applications of Synthetic Seeds

Synthetic seed technology imitates natural seed production and can be used as the same also. It can be used to propagate and conserve endangered and threatened plant species (Fig. 1).

4.1 Reintroduction

According to the IUCN (1987), reintroduction is the 'intentional movement of an organism into part of its native range from which it has disappeared or become extirpated in historic times as a result of human activities or natural catastrophes'. Endangered plant species can be reintroduced in their natural habitat using synthetic seeds; however, it is important to take into consideration the geographic characteristics of the areas to be repopulated and to avoid outbreeding and inbreeding depression. Several endangered plant species have been multiplied in in vitro conditions and reintroduced including *Cochlearia bavarica* Vogt (Kaulfuß and Reisch 2017), *Castilleja levisecta* Greenm. (Salama et al. 2018), *Woodsia ilvensis* (L.) R.Br. (Aguraiuja 2011), and *Renanthera imschootiana* Rolfe (Wu et al. 2014). The use of synthetic seed technology for reintroduction will make this process faster as synthetic seed preparation takes lesser time as compared to plantlet development and is efficient due to ease of transportation.

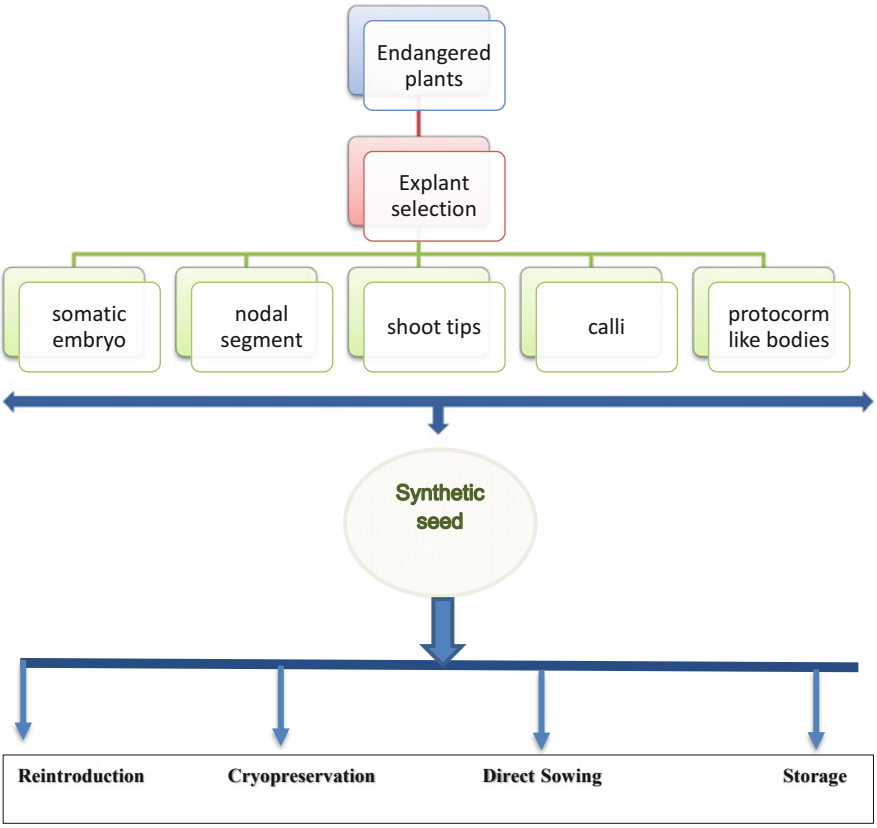

Fig. 1 Flowchart depicting the use of synthetic seed technology for conservation of endangered plants

4.2 Direct Sowing

Synthetic seeds do not require an acclimatisation period which is mandatory for micropropagated plants (Gantait and Kundu 2017). Direct planting has been successful in several species including alfalfa (*Medicago sativa* L., cv. Regen S, clone RA_3) (Fujii et al. 1992), *Dalbergia sissoo* Roxb. (Chand and Singh 2004), *Phyllanthus amarus* L. (Singh et al. 2006), and *Erythrina variegata* L. (Javed et al. 2017). The synthetic seeds, including those of medicinal and endangered plants, can be directly sown in soil or planting substrates like sand, soilrite, perlite, and vermicompost (Mandal et al. 2000) for higher germination and survival rates.

4.3 Storage and Transport

Seed banks are normally used to store the seed of endangered plant species around the world. However, seed banking requires periodic assessment of germination and viability of the stored accessions. The germination and viability of stored germplasm are often influenced by taxonomic and plant specific characteristics (Godefroid et al. 2010). Synthetic seeds can also be stored at lower (−4 °C for cucumber; Tabassum et al. 2010) or higher temperature (25 °C for hybrid orchid *Aranda* Wan Chark Kuan 'Blue' × *Vanda coerulea* Grifft. ex. Lindl.; Gantait and Sinniah 2013). Apart from ease of storage, synthetic seeds can be transported easily around the globe, and germplasm can be exchanged easily due to small size and lighter weight (Gantait et al. 2017) compared to bulky plantlets and seedlings.

4.4 Dried Somatic Embryo Production and Encapsulation in Endangered Plants

Synthetic seeds can be developed by encapsulation of hydrated as well as dried somatic embryo. The dried somatic embryos are produced by treating the somatic embryo with ABA, nutrient stress, environmental stress, and air-drying. The advantages of dried somatic embryo over the hydrated ones are desiccation tolerance, increased germination efficiency, and longer storability. In *Medicago sativa* L. (McKersie et al. 1989), dried somatic embryos have been developed by ABA treatment and were stored with no loss of viability for 8 months; however, loss of vigour was observed. Winkelmann et al. (2004) developed dried somatic embryos in *Cyclamen persicum* Mill. by drying somatic embryos at different levels of relative humidity generated by desiccators filled with saturated salt solution. The dried somatic embryos showed high germination, bigger tubers, and better cotyledons. In grapevine, the dehydrated embryos were stored for 42 months and 90% germinated into plantlets (Jayasankar et al. 2005). In the case of Norway and Serbian spruce, partly dried somatic embryo showed improved radicle growth and germination (Hazubska-Przybył et al. 2015). Development of dried somatic embryo for endangered plant species will aid in better synthetic seed production, longer storage period, ease of transport, and transplantation in natural habitats.

4.5 Cryopreservation of Synthetic Seed

The storage of biological material at ultra-low temperature (−196 °C) at which all cellular divisions and metabolic processes are stopped for long-term conservation is known as cryopreservation (Engelmann 2004). The technique of cryopreservation employs several methods—like classical method of freeze-induced dehydration,

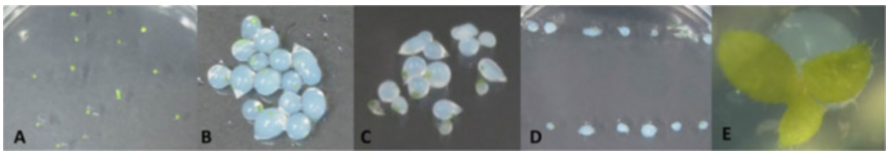

Fig. 2 Potential steps for synthetic seed development for in vitro meristem of *Draba yukonensis* (**a**) using sodium alginate beads (**b**) and dehydrating the beads containing meristem (**c**) for storage or cryostorage. The synthetic seeds cultured on the MS basal medium (**d**) for shoot growth development (**e**) under in vitro condition (Saxena et al. unpublished)

vitrification of internal solutes, encapsulation–dehydration, encapsulation–vitrification, droplet vitrification, etc. The technique of encapsulation–dehydration is an extension of the artificial seed technology (Fig. 2a–e). In this technique, the explants (meristem, zygotic or somatic embryo) are encapsulated in alginate beads and pre-cultured in liquid medium enriched with sucrose for 1–7 days. Later encapsulated explants undergo partial desiccation in the air current of a laminar airflow cabinet or with silica gel down to a water content around 20% (fresh weight) and are then frozen rapidly. This method has high survival rates, and growth recovery of cryopreserved samples is faster and direct (Engelmann 2000). It avoids the use of cryo-solutions on explants. Encapsulation of explants in an alginate bead has several advantages such as higher availability of mineral and hormonal nutrients (Fabre and Dereuddre 1990), ease of cryopreservation of desiccation-sensitive material (Redenbaugh 1993), and slow release of endogenous substances to outside media due to the presence of bead (Flachsland et al. 2006). In a study conducted on *Ribes nigrum* cultivar Ben More (Benson et al. 1996), the alginate-encapsulated/air-evaporated desiccated meristems had stable glass transition profiles upon cryopreservation. Several plant species have been successfully encapsulated and cryopreserved including *Solanum phureja* Juz. and Bukasov (Fabre and Dereuddre 1990), protocorms of *Cleisostoma arietinum* (Rchb.f.) Garay (Maneerattanarungroj et al. 2007) and *Vanda coerulea* (Jitsopakul et al. 2008), protocorm like bodies (PLBs) of *Phalaenopsis bellina* (Rchb.f.) Christenson (Khoddamzadeh et al. 2011), and *Dendrobium* Sonia-17 (Subramaniam et al. 2011). The synthetic seeds of endangered plants like *Centaurium rigualii* Esteve (Gonza et al. 1997), *Cosmos atrosanguineus* (Hook.) Voss (Wilkinson et al. 2003), *Betula lenta* L. (Rathwell et al. 2016), and *Castilleja levisecta* Greenm. (Salama et al. 2018) can be cryopreserved and used for long-term conservation.

5 Synthetic Seeds in Endangered and Rare Plants: Examples

The following selected examples illustrate the usefulness of synthetic seeds for propagation and long-term conservation.

5.1 *Ipsea malabarica (Reichb. f.) J. D. Hook*

Ipsea malabarica (Reichb. f.) J. D. Hook is an endemic and endangered orchid which belongs to family Orchidaceae of the Western Ghats of Kerala, India. In this species, reproduction is obstructed by slow rhizome propagation, and seed propagation relies on mycorrhizal association (Martin and Pradeep 2003). Martin (2003) coated bulbs in half-strength MS medium (devoid of $CaCl_2$) fortified with 6.97 mM KN containing 3.0% (w/v) sodium alginate and 3.0% sucrose. The treated bulbs were dropped into sterilised $CaCl_2$ (0.7%) solution for 30 min. The encapsulated bulbs were cultured both on half-strength MS agar medium with or without 6.97 mM KN, and 100% conversion was recorded.

5.2 *Swertia chirayita (Roxb. Ex Fleming) H. Karst*

Swertia chirayita (Roxb. ex Fleming) H. Karst is an annual/biennial herb which belongs to family Gentianaceae and is categorised as critically endangered by the IUCN (Joshi and Dhawan 2005). It is popular as a medicinal plant due to its wide usage in traditional medicines (Kumar and Van Staden 2016). The plant is distributed in regions of Himalaya, Kashmir, Shillong, Bhutan, and Khasi hills. A drastic reduction in the population has resulted from over-exploitation of plants by local people and traders. Moreover, the life cycle of the plant is hindered due to low seed viability and germination rates (Joshi and Dhawan 2007). Joshi and Dhawan (2007) developed a protocol for axillary multiplication from 4-week-old seedling-derived nodal explants by supplementing MS medium with 4 μM BA and 1.5 μM 2iP. The shoots can be potential explants and can be encapsulated. Kumar and Chandra (2014) developed a protocol for somatic embryogenesis from in vivo leaf explants by supplementing MS media with 0.5 mg/L 2,4-D and 0.5 mg/L KN which resulted in 76% of embryogenic callus. Torpedo stage embryos were encapsulated in sodium alginate (4% W/V) gel, dropped into 100 mM calcium chloride ($CaCl_2 \cdot 2H_2O$) solution to produce synthetic seed. The most effective germination medium was MS medium supplemented with 1.0 mg/L BA and 0.5 mg/L NAA with a success rate of 84%. The highest plantlet survival rate of 80% was obtained in soilrite and sand in 1:2 ratio. The plantlets were successfully acclimatised to ex vitro conditions.

5.3 *Tylophora indica (Burm. Fil.) Merrill*

Tylophora indica (Burm. Fil.) Merrill is a threatened medicinal herb which belongs to the Apocynaceae family and is native to India. The root and leaves of the plant are source of alkaloids such as tylophorine, tylophorinine, tylophorinidine, and tylophorindine. The key bioactive agent is tylophorine which is anti-inflammatory

and anti-asthmatic (Kaur and Singh 2012). The plant suffers from overharvesting pressure and slow multiplication rate due to poor seed viability, low germination rate, limited fruit set, and small number of propagules (Thomas and Philip 2005). Faisal and Anis (2007) produced artificial seeds by encapsulating nodal segments in 3.0% (w/v) sodium alginate and 100 mM $CaCl_2 \cdot 2H_2O$. The maximum conversion rate was achieved on MS medium containing 2.5 μM BA and 0.5 μM NAA. Successful conversion and plantlet development were observed from encapsulated nodal segments stored at 4 °C for 8 weeks consecutively. Direct sowing of encapsulated nodal segment on Soilrite™ moistened with 1/2 MS salts led to a high conversion rate. The plants regenerated from encapsulated nodal segments were successfully hardened, acclimatised, and established in soil. Gantait et al. (2017) used nodal segments and prepared artificial seeds using 75 mM calcium chloride ($CaCl_2 \cdot 2H_2O$) and 3.0% (w/v) sodium alginate. The artificial seeds were stored at (5 ± 1) °C, (15 ± 1) °C, and (25 ± 1) °C, and highest frequency of conversion (90%) was obtained at a (15 ± 1) °C after 15 days and 70% after 30 days of storage.

5.4 *Gentiana lutea L.*

Gentiana lutea L. is a medicinal plant which belongs to family *Gentianaceae* with gastro-protective effects (Aberham et al. 2011; Niiho et al. 2006). The roots of this plant are used in food products and also traditional medicine to stimulate appetite and for better digestion (Leung 1980). The plant is herbaceous perennial and grows in Europe and Asia. The plant has slow growth habit and produces dormant seed (González-López and Casquero 2014). The species is assessed as least concern (LC) in the IUCN European Red List (Bilz et al. 2011). Holobiuc and Catana (2012) developed synthetic seeds by encapsulating somatic embryos in 3.0% sodium alginate and 100 mM $CaCl_2$. The optimum temperature for synthetic seed preservation was 10 °C in the growth room for several months.

5.5 *Bacopa monnieri (L.) Wettst.*

Bacopa monnieri (L.) Wettst. is a perennial herb from family *Scrophulariaceae* and grows in the wetlands of southern and eastern India, Australia, Europe, Africa, Asia, and North and South America. The plant and plant extracts have been used in traditional medicines as a sedative, anti-inflammatory, analgesic, antipyretic, and antiepileptic agent (Russo and Borrelli 2005). Demand for the plant is very high with the pharmaceutical industry using around 6600 tonnes/year in India (National Medicinal Plant Board 2004) which resulted in drastic depletion of the wild plant population. The life cycle of the plant is also limited by poor seed viability (60 days), seedling death at the two-leaf stage, and slow vegetative propagation (Tiwari et al. 2001). The growth habit of the plant is habitat specific as it prefers open spaces (Shah

1965). The plant is categorised as least concern in the IUCN Red List (Lansdown et al. 2013). Khilwani et al. (2016) developed a protocol by using leaf explants for somatic embryogenesis, shoot organogenesis, and encapsulation of the embryos and shoots in order to produce synthetic seeds. Shoot tips and somatic embryos were encapsulated in sodium alginate (3.0%, w/v) and 125 mM calcium chloride ($CaCl_2 \cdot 2H_2O$). The synthetic seeds were analysed for short-term storage by storing at 4 °C and 25 °C (room temperature) for 140 days. The artificial seeds retained viability after 140 days of storage at both temperatures, but the germination of encapsulated somatic embryos was higher when stored at 25 °C. However, the encapsulated apical shoot buds failed to germinate after 40 days when stored at 4 °C. Sharma et al. (2012) encapsulated nodal shoots using 2.5% sodium alginate and 100 Mm $CaCl_2 \cdot 2H_2O$. The highest conversion rate (86.67%) was achieved on MS medium supplemented with 1.0 mg/L BAP after 6–8 weeks. The regenerated plantlets were hardened, acclimatised, and established under net house conditions.

6 Future Prospects: Technology Improvement and Applications

The synthetic seed technology can be used to save endangered and rare plant species by following methods:

6.1 Development of Synthetic Seeds to Generate Variability in Endangered Plant Species

Plant species categorised as endangered and rare are resorted to that status due to several anthropogenic, environmental, and physiological factors. Loss of diversity and variation is also one of them. The central theme of conservation biology is to maintain genetic variation in endangered plants (Frankel and Soulé 1981). Genetic variation is indispensable for long-term survival of endangered plant species (Frankel and Frankel 1970). The synthetic seed can be used as source of potential variation in rare and endangered plant species just like naturally produced seeds. The variation originating in cell and tissue culture is described as ‘somaclonal variation’ (Larkin and Scowcroft 1981). However, the definition has evolved with time and application of the same. Morrison et al. (1988) described somaclonal variation ‘as genetic variation observed among progeny of plants regenerated from somatic cells cultured in vitro’. Tapingkae et al. (2012) emphasised on the practical aspect of the phenomenon ‘Somaclonal variation is a valuable tool in plant breeding, wherein variation in tissue culture regenerated plants from somatic cells can be used in the development of crops with novel traits’. The mechanism of variation can be genetic and epigenetic (Gao et al. 2009). The genetic factors (pre-existing variation) can arise due to use of

chimera as explant (George 1993; Kunitake et al. 1995), chromosomal anomalies (Lee and Phillips 1988; Mujib et al. 2007), cell cycle abnormalities (Larkin and Scowcroft 1981; Lee and Phillips 1988), and active transposons (Pietsch and Anderson 2007; Barret et al. 2006). The epigenetic factors (can be tissue culture induced) are type of explants (Israeli et al. 1996; Sharma et al. 2007), source of explants (Kawiak and Łojkowska 2004; Chuang et al. 2009), genotype (Etienne and Bertrand 2003; Bordallo et al. 2004), plant growth regulators (Peschke and Phillips 1992; Giménez et al. 2001), number of subculture (Rodrigues et al. 1997), duration of culture (Reuveni and Israeli 1989; Bairu et al. 2006), and effect of stress (Lee and Phillips 1988; Halim et al. 2018). Somaclonal variations have been observed and exploited in different species. Krishnamurthi and Tlaskal (1974) developed Fiji disease-resistant *Saccharum officinarum* L. somaclones. Shepard et al. (1980) developed potato somaclones with tubers of uniform skin colour, size, and shape, and they increased fruit production and disease resistance to early blight. In rice, Joshi and Rao (2009) developed submergence tolerant somaclones. Somaclonal variation has also been reported in endangered and rare plant species in vitro culture. Dey et al. (2015) developed somaclones in *Cymbopogon winterianus* Jowitt. and observed significant variation for agronomic traits like plant height, diameter of bush, number of tiller/clump, number of leaves/clump, leaf length, leaf breadth, weight of leaves, and essential oil content and quality. The somaclones were subjected to stability analysis, and the polymorphism was confirmed by random amplified polymorphic DNA (RAPD) analysis. Slazak et al. (2015) developed somaclones in an endangered medicinal plant *Viola uliginosa* Besser with higher cyclotide production. In this study, leaf and petiole were cultured on MS media supplemented with TDZ (0.5 or 1 mg L^{-1}) or with equal concentrations (2 mg L^{-1}) of KN and 2,4-D, followed by callus transfer on 1 mg L^{-1} TDZ. The regenerants had different ploidy level as compared to diploid mother plant; majority of them were tetraploid. The tetraploid plants had higher production of cyclotides. The morphological variation in somaclones has also been studied in some rare plant species. Isabel et al. (1996) observed four variegated phenotypes in plants developed from somatic embryos of *Picea glauca* (Moench) Voss (least concern). The variation was due to chlorophyll deficiency in leaves and the presence of green and white cells. The variegated phenotypes were screened with 250 RAPD markers and correlated with one of them. Tremblay et al. (1999) studied morphological variation in plants from somatic embryos of two pine species, *Picea mariana* (Mill.) Britton, Sterns and Poggenburg and *Picea glauca* (Mill.) Britton. The morphological variation was categorised into nine types based on plant architecture: dwarf, bushy, normal needle hooked stem, reduced height with thick short needles, needle fasciation, abnormality in tree architecture, variegated phenotype, plants with an overall regular morphology but smaller than normal plants and plagiotropic plants. Two separate phenotypes dwarf and reduced height with thick short needles had aneuploid cells indicating chromosomal instability. The somaclones with desirable variation can be encapsulated and used as synthetic seeds. However, the somaclonal variation has to be screened for stability and heritability for successful creation and maintenance of genetic variation in endangered and rare plants.

6.2 Use of Synthetic Seeds for Mass Reforestation Programme

Endangered and non-endangered forest tree species many a times have problems in seed production and germination. A period of 20–30 years is needed to produce seeds for reforestation (Gupta and Kreitinger 1993). Synthetic seeds can be produced in a shorter period of time and can be transported and planted in barren land and forest area. Synthetic seeds have been reported in forest tress; some examples are *Jacaranda mimosaefolia* D. Don (vulnerable) (Maruyama et al. 2007) and *Pinus patula* Scheide et Deppe (least concern) (Malabadi and Van Staden 2005).

6.3 Integrated and Combined Strategy for Production of Synthetic Seed of Endangered Plants and Rearing the Animal Species with Which It Has Mutualism Interaction

Flora and fauna share a close relationship with each other in an ecosystem, and endangered plant species are no exception. Several endangered plants depend on animals to complete their life cycle and spread their population. In order to establish a balanced ecosystem, it is required that apart from planting and transplanting endangered plants and sowing synthetic seed, the animal which helps in the survival of the plant should also be introduced in the area of planting. Some of the examples are gecko (*Phelsuma cepediana* Milbert) which is involved in pollination and seed dispersal of endangered *Roussea simplex* Sm. The relationship is disrupted by invasive ants *Technomyrmex albipes* Smith which feed on nectar and fruit pulp of the plant and limit the visitation rate of the lizard to the flowers (Hansen and Müller 2009). A primate yellow-breasted capuchin (*Sapajus xanthosternos* Wied.) (critically endangered) is responsible for seed dispersal for 23 endemic and 3 endangered plants, and it has a significant role in seed dispersal of *Manilkara* sp. (Canale et al. 2016). Unfortunately, many frugivores are facing threat of hunting and decrease in population. Traveset and Riera (2005) studied the seed dispersal in a perennial shrub, *Daphne rodriguezii* (Texidor), by a frugivorous lizard *Podarcis lilfordi* Günther and found that the lizard is facing extinction due to carnivorous mammals like weasels, pine martens, genets, and cats. It is therefore important to consider the factors that influence the entire life cycle of a plant in its natural environment for effective strategies of long-term conservation.

7 Conclusion

In spite of the fact that synthetic seed technology has existed since 1985 and has been widely used for production of medicinal, forest, and vegetable species, the full potential of this technology has not been utilised for the conservation of rare and endangered plant species. The available literature suggests that the micropropagation and somatic embryogenesis protocols of several endangered plants exist, but still there is lack of synthetic seed preparation and utilisation. The synthetic seeds in endangered plant species can be used for production, conservation, cryopreservation, and reintroduction, thus saving them from extinction.

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Synseed: A New Trend in Seed Technology

Buhara Yücesan

Abstract Synseed technology is a growing trend in innovative and sustainable agriculture applications. It provides a promising method based on encapsulation of embryonic (somatic embryos) or non-embryonic tissues (i.e., shoot tips, shoot buds, microshoots, nodal segments, protocorms, etc.) in a gel-like matrix for the massive plant production. This approach can be generalized for the commercial production when the techniques minimize the production costs of elite plant genotypes in case of conservation and delivery. For the last 20 years, plant tissue culture researchers have focused on practical implementations of synseed technology which are yet to be solved such as preference of chemical composition for encapsulation, sowing and subsequent regrowth efficiency, and optimization of storage conditions prior to the plant material transportation. This chapter focuses on the basics of synseed production underlying the practical applications, its achievements, and limitations, which might provide a new insight for the farmers of the twenty-first century.

Keywords Synseed · Encapsulation · Sodium alginate · Artificial seed · Seed technology

1 Development of Seed Technology

A seed is formed after fertilization of egg in a protective coating in vascular plants. It allows plants to propagate across great distances, borne by wind or water or sometimes stuck to other organisms for the transportation. Seed provides a superior advantage to the diploid embryo which is readily found in a protective coat and storage tissue. From this view of evolutionary perspective, the need of seed for the plant propagation is as old as the history of mankind. Man has become so utterly dependent on the plants; thus

B. Yücesan (✉)
Department of Seed Science and Technology, Bolu Abant İzzet Baysal University, Bolu, Turkey

Department of Horticultural Science, North Carolina State University, Raleigh, NC, USA
e-mail: buhara@ibu.edu.tr

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he grows for food, fights for food, and dies for food. This interaction did not domesticate the man only as a process of transition from a lifestyle of hunting and gathering to the agricultural society with a settlement making larger populations but also all those of plants fully domesticated have not survived without the aid of human since Neolithic period (Harari 2015). This can also be associated with a first agricultural revolution in human cultures that paved the way of industrial revolution in Europe starting from Britain in the eighteenth century. New farming techniques eventually increased the population as well as prosperity in developed countries. However, more efforts were needed in agriculture for satisfactory food production; the growing populations could not meet the need for adequate nutrient for their life. At this point, green revolution was glittered in the 1960s; seed technology with innovative fertilizer and irrigation methods pioneered the high yielding variety of seeds. Moreover, Japanese scientists Toshio Murashige and Folke Skoog published a well-documented tissue culture protocol on tobacco plant, and their approaches inspired the researchers for the plant propagation under in vitro conditions (Murashige and Skoog 1962). Upon a simple literature survey in the Internet, it seems clear that more than 10,000 plants have been propagated through tissue culture techniques as yet, and those reports on plant propagation differ in either the way or techniques used or just a snapshot of trending topics.

Synseed, artificial or synthetic seed production was coined for the first time by Toshio Murashige as an "encapsulation of a somatic embryo" (Murashige 1977). Today, when taken into account the topic of "synthetic seed" as a keyword refined by "encapsulation" from the Web of Science core collection (WOS 2018) covering a wide variety of indexes, around 250 results are shown, and majority of the studies have been reported in the last decade (covering 66% of total publications; see Fig. 1).

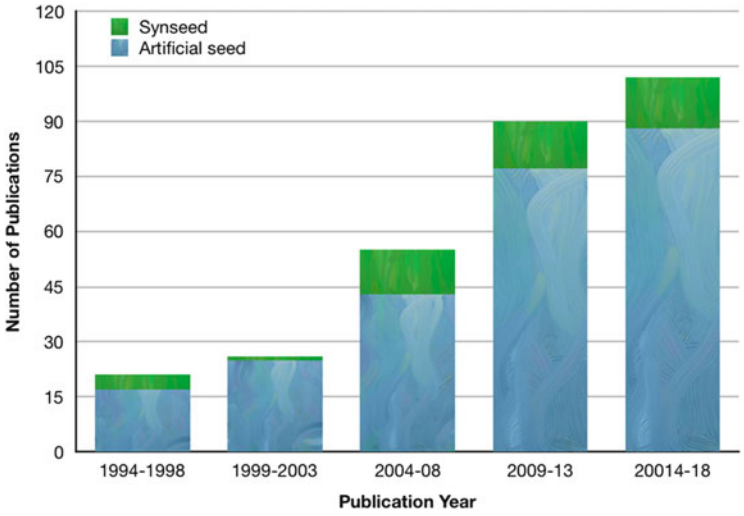

Fig. 1 Publications in the last quarter of the century that focused on synseed technology with the preference of the terms, *synseed* in green bars versus *artificial seed* in blue bars [Retrieved from the Web of Science Core Collection (WOS 2018). Index coverages are SCI-expanded, SSCI, A&HCI, CPCI-S, CPCI-SSH, BKCI-S, BKCI-SSH, ESCI, CCR-expanded, IC]

However, synseed versus artificial seed emerges more as a new term with only 44 publications (Fig. 1). The idea behind this innovative seed production was based on engineering the tissue for the practical use of plant production. Thus, this approach can be generalized for the commercial production when the techniques minimize the production costs of value-added plant species. In this context, a real seed that involves a zygotic embryo was mimicked on a meristematic tissue which enables diploid embryo production, and these somatic embryos were beaded into a polymer matrix to sustain further growth and development as seen in nature. Synseed production could also be achieved by in vitro-derived propagules without prefering direct use of somatic embryos, since many plants are recalcitrant to somatic embryo production under in vitro conditions. Especially, non-embryonic tissues, such as shoot tips, shoot buds, microshoots, nodal segments, protocorms, etc., can also be used in synseed production with an ability to be converted into a complete plant under in vitro or ex vitro conditions (Fig. 2).

2 Plant Tissue Culture Techniques for the Synseed Production

2.1 Somatic Embryogenesis

Somatic embryos are of great potential for the synseed production, since they have bipolar differentiation during development resulting in a radicle and plume axis in a single step as compared to the non-embryonic tissues having unipolar differentiation at the initiation step. It means that root formation occurs prior to the shoot organogenesis, and complete regeneration may take longer period than somatic embryogenesis. In theory, plant cells can initiate embryo development in two main ways: regaining embryogenic cell identity via induction and losing vegetative/somatic cell identity via reversion (Fehér et al. 2016). In other words, these plant cells are not inherently embryogenic but become embryogenic in response to external or internal signals which are mostly regulated by auxin concentrations as well as epigenetic factors under certain conditions. The plants species which are capable of producing somatic embryos keep their regenerative capacity for a long time by passing intervening callus stage, thus making a clonal propagation genetically stable. Aitken-Christie et al. (1994) improved the idea of encapsulating the somatic embryo to such an extent that synseed technology was defined as artificially encapsulated somatic embryos, sprouts or other tissues that may be used for in vitro or ex vitro culture conditions. Actually somatic embryos can be used directly for large-scale planting. This might be why Toshio Murashige proposed the term of encapsulation of somatic embryos in 1978 (Murashige 1977). To achieve this, a protective layer that covers the somatic embryo must be of a certain quality: (1) it must be non-damaging to the embryos, (2) it must be protective but also sufficiently durable for the handling, and (3) it must have a good enough matrix composition that

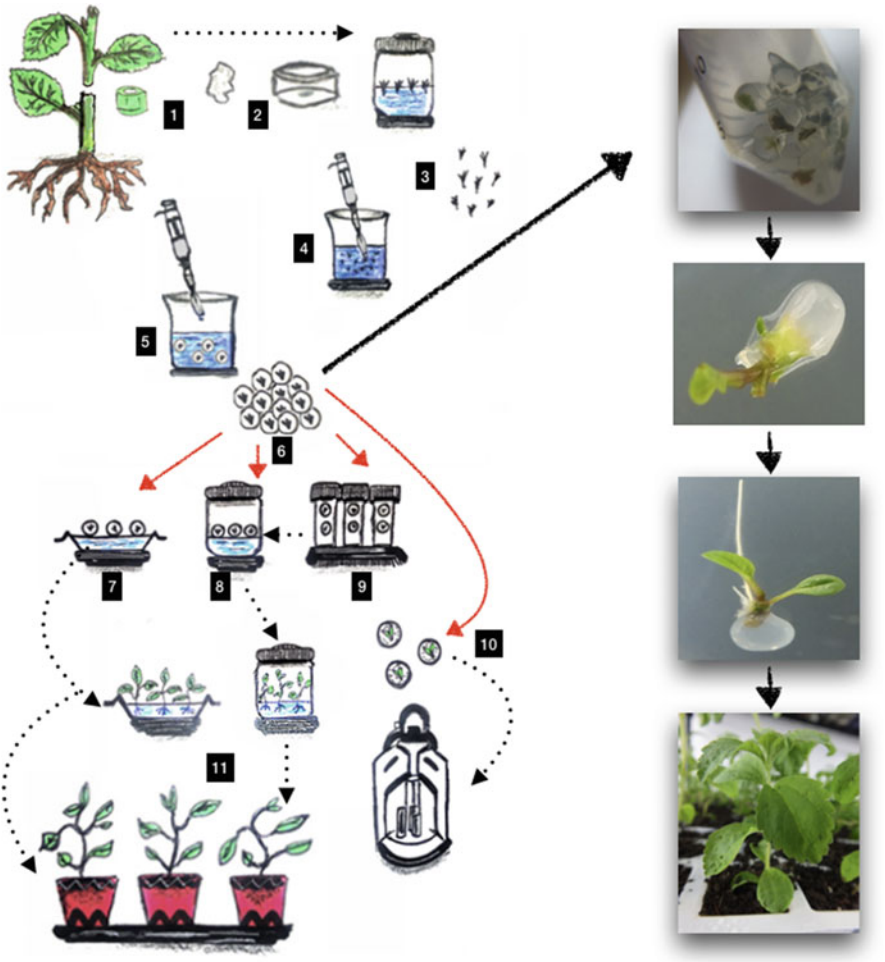

Fig. 2 Schematic representation of synseed technology. Explant selection from a plantlet (1) or callus tissue (2) for the somatic embryo production. Depending on the regeneration response, non-embryonic tissues or somatic embryos are added into a polyelectrolyte solution (i.e., sodium alginate with or without growth medium composition and respective additives) and sucked up by a micropipette with a cut pipette tip (4), transferring the tissue into a crosslink solution (i.e., calcium chloride) for the gelation from outside to inside (5). After polymerization step, the bead-like synseeds (6) are either directly transferred into an open non-axenic sowing medium (7) or transferred into growth media under in vitro conditions (8). For the storage purpose, short-term (9) or long-term (i.e., cryopreservation) conservation of synseeds in liquid nitrogen (10) can be applicable depending on the future application(s) (11). Photographs at the right margin show the steps from the synseed production to the complete plantlet formation in *Stevia rebaudiana* (Photographs from Buhara Yücesan; the diagram showing the steps of synseed technology was drawn by Ece Tıpyardım)

contains nutrients, growth regulators, etc.; high quality of the embryo inside of the beads is promptly needed for the success of germination. As to the germination pattern, somatic embryos generally perform poor germination as compared to their convertibility into whole plantlets. This is also an observable pattern for the seed embryos in nature, since the reserve food materials such as proteins and secondary metabolites impart desiccation tolerance to the embryo, thus promoting normal development to be prepared for the germination. Accumulation of ABA at this stage is critical for the prevention of precocious germination; instead it facilitates embryo development by suppressing the secondary embryogenesis. For the synseed production, secondary embryo production can be more useful over primary ones, because newly formed somatic embryos are formed in large quantities from primary somatic embryos. Therefore, high commercialization with high production rate without the need of explant source might be feasible for the selected lines of secondary embryos in a short period.

2.2 Non-embryonic Meristematic Tissue Production

Synseed production is not only based on somatic embryos as mentioned before, but also non-embryonic tissues might replace the success of somatic embryos in novel technology. These non-embryonic tissues or explants such as shoot tips, nodal segments, callus tissues derived from cotyledonary, mature leaves, and internodes are widely used in synseed production (Table 2). In contrast to somatic embryos, non-embryonic meristems undergo a unipolar differentiation; thus further root meristems are needed to induce the root system before or after encapsulation depending on the suitability of the explants for the tissue culture system as well as the synseed production (Ara et al. 2000; Sharma et al. 2013; Gantait and Kundu 2017). Yücesan et al. (2015) reported that nodal explants of goldenberry (*Physalis peruviana*) were suitable for the encapsulation, and those of synseeds induced root formation without using any growth regulator in either encapsulation matrix or sowing medium. However, Nishitha et al. (2006) offered a protocol for the synseeds of Bengal creeper using an auxin for the root induction in encapsulation matrix (see Table 2). The best way is to understand the suitability of the explants for the synseed production; regeneration of a certain plant species should be well-documented in order to achieve a simultaneous shoot and root induction of capsulated explants on sowing media (see the steps in Fig. 2). For healthy plant regeneration, shoot tips eliminate the plant pathogens, thus providing an efficient explant source for synseed capsulation. Nodal segments are of great potential for meristematic activity, and they respond effectively to the plant growth regulators producing multiple shoots or callus tissue depending on medium formulation. Protocorm-like bodies (PLBs) resemble somatic embryos, and the production of PLBs has been reported mainly in orchid species (Lee et al. 2007). Although synseed production is limited in this tissue type, there are reports on some ornamental plants, Cymbidium Twilight Moon 'Day Light' (Teixeira da Silva, 2012) and Dendrobium Shavin White (Bustam et al. 2013).

3 The Types of Encapsulation in Synseed Production

There are simply two types of synthetic seeds, desiccated synthetic and hydrated seeds. The first attempt on desiccated synseed production was established by Kitto and Janick (1985) using somatic embryos of carrot (*Daucus carota*). After encapsulation of the multiple somatic embryos in a water-soluble polyoxyethylene glycol (Polyox), these embryos were desiccated overnight. This technique was also developed by a group of scientist, and they applied PEG-based mixture for carrot somatic embryos and embryogenic callus. After that, drying out was achieved on Teflon surface overnight in running laminar flow cabinet. Desiccated synseeds stored at different temperatures was then rehydrated in culture media to check viability of somatic embryos. It seems clear that for an efficient desiccation synthetic seed production, somatic embryos should be desiccation tolerant. Ara et al. (2000) reported a clear insight about the achievement of desiccation through storage applications at different periods in growth chambers with relatively low humidity, and condition of petri plates without parafilm sealing let the synseeds overnight to be dried. Sundararaj et al. (2010) reported the synseeds transferred to the liquid medium containing various sucrose as an example for the dehydration that resulted in high recovery at 0.5 M sucrose. Desiccated synseed production bears some limitation unless embryos are coated with a protective and nutritious layer, especially at the early stage of regrowth. As seen in the real seed coats (testa layer of the seeds), artificial layer that mimics the seed coat must be non-toxic, non-aqueous but sufficiently soft to facilitate the emergence of shoot and root primordia (Pond and Cameron 2003). For the second group of synseeds, they are produced by encapsulating embryonic or non-embryonic tissues in a hydrogel matrix (see Table 2). Encapsulation is achieved by an ion exchange process between calcium and sodium ions that form an alginate bead (calcium alginate) in case of synseed production. However, this technology is not new and has already been used in food industry widely for 50 years (Gibbs et al. 1999). The idea of encapsulation is based on protecting the items in a gel capsule from the environment. From this point of view, plant tissues can be encapsulated in a single- or double-layer hydrogel encapsulation.

3.1 Single-Layer or Double-Layer Encapsulation

Single-layer encapsulation is the simplest and widely used layering process for which varying concentrations of polymer matrix or hydrogel such sodium alginate (up to 5.0%, w/v) are largely used. For single-layer encapsulation, two different solutions are used, calcium chloride ($CaCl_2$) as crosslink solution and sodium alginate as a polyelectrolyte in which plant tissues are readily suspended. Subsequent interaction of these aforesaid solutions at various concentrations results in an ion exchange between calcium and sodium in a certain time for the polymerization (ranging between 15 and 50 min; see Tables 1 and 2). Plant tissues are immobilized

Table 1 Synseed production using somatic embryos based on encapsulation matrix, duration of capsulation, and conversion medium with a percentage of recovery from selected literature

Plant name	Encapsulation matrix	Concentration of $CaCl_2$ (%)	Polymerization time (min)	Regrowth medium	Recovery (%)	References
Banana (*Musa* spp.)	5% NaAlg in MS	2.5	30	MS	66	Ganapathi et al. (2001)
Paulownia tree (*Paulownia elongata*)	3% NaAlg in MS	2.2	30	Peat/perlite (1:1)	53	İpekci and Gozukirmizi (2003)
Patula pine (*Pinus patula*)	2.5% NaAlg + DCR basal salts	4.4	5	½ DCM	89	Malabadi and van Staden (2005)
Guava (*Psidium guajava*)	2% NaAlg	4.4	20–30	MS medium	92	Rai et al. (2008)
Butterfly pea (*Clitoria ternatea*)	4% NaAlg + MS + 3% sucrose + 1 mg/L	4.4	45	MS supplemented with 2 mg/L BA + 0.5 mg/L NAA	92	Kumar and Thomas (2012)
Periwinkle (*Catharanthus roseus*)	2.5% NaAlg + 3% sucrose	4.4	15	MS medium + 0.25 mg/L NAA and BA	84	Maqsood et al. (2012)
Grape hyacinths (*Muscari armeniacum*)	1.5% NaAlg	1	20	Soil	87	Yücesan et al. (2014)
Squill (*Ledebouria revoluta*)	1.5% NaAlg	6.6	15	MS	96	Haque and Ghosh (2016)
Giant squill (*Drimiopsis kirkii*)	1.0% NaAlg	3	15	MS	93	Haque and Ghosh (2014)

(continued)

Table 1 (continued)

Plant name	Encapsulation matrix	Concentration of $CaCl_2$ (%)	Polymerization time (min)	Regrowth medium	Recovery (%)	References
Rice (*Oryza sativa*)	4% NaAlg + MS (without $CaCl_2$) + 3% sucrose + PGR(0.5 mg/L NAA, 0.5 mg/L IAA, 2 mg/L BA) + antibiotics (1 mg/l bavistin, 1 mg/L streptomycin) + 1.25% activated carbon	1.5	10	Whatman filter paper irrigated with ½ MS + vitamins + 2% sucrose	47	Arun Kumar et al. (2005)

NaAlg sodium alginate, *BA* 6-benzyladenine, *IAA* indole-3-acetic acid, *IBA* indole-3-butyric acid, *MS medium* Murashige and Skoog's medium (Murashige and Skoog 1962), *PGR* plant growth regulators, *NAA* α-naphthalene acetic acid

Table 2 Synseed production using non-embryonic meristems based on encapsulation matrix, duration of capsulation, and conversion medium with a percentage of recovery from selected literature

Plant name	Explant type	Encapsulation matrix	Concentration of $CaCl_2$ (%)	Polymerization time (min)	Regrowth medium	Recovery (%)	References
Kiwifruit (*Actinidia deliciosa*)	Apical meristems, axillary buds	2.5% NaAlg + QL + 2.5% sucrose +1 mg/L BAP and GA_3	4.4	30	½ QL medium	Max 57% depending on the medium	Adriani et al. (2000)
Adusa (*Adhatoda vasica*)	Shoot bud	4% NaAlg + B5 + 1.0 mg/L KIN + 50 mg/L PG	1.1	20	B5 medium	67%	Anand and Bansal (2002)
Malabar daffodil orchid (*Ipsea malabarica*)	Bulbs	3% NaAlg + ½ MS medium + 6.97 μM KIN	0.7	30	½ MS medium with or without 1.5 mg/L KIN	100%	Martin (2003)
Orchid spp. (*Dendrobium* sp., *Oncidium* sp. *Cattleya* sp.)	Protocorm-like body structures	3% NaAlg + ¾ MS + 0.1 mg/L BA and NAA (for *Dendrobium*), 0.5 mg/L NAA (for *Oncidium*), and 1.0 mg/L (for *Cattleya*)	3	30	MS + 0.1 mg/L BA + 0.1 mg/L 0.5 mg/L NAA (for *Dendrobium*) MS + 0.5 mg/L NAA (for *Oncidium*) MS + 1.0 mg/L NAA (for *Cattleya*)	100% for three spp.	Saiprasad and Polisetty (2003)
Dalbergia sissoo (sissoo)	Nodal segments	3% NaAlg + ½ MS	6.6	20	½ MS	85%	Chand and Singh (2004)
Dendranthema × *grandiflora* (chrysanthemum)	Nodal segments	3% NaAlg + MS + 1.0 mg/L IAA	6.6	30	Perlite moistened with MS	50%	Pinker and Abdel-Rahman (2005)
Bengal creeper (*Chonemorpha grandiflora*)	Shoot tip	3% NaAlg + ½ MS (except $CaCl_2$) + 0.1 mg/L IBA + 11.7 μM silver nitrate	2.2	30	½ MS	95%	Nishitha et al. (2006)

(continued)

Table 2 (continued)

Plant name	Explant type	Encapsulation matrix	Concentration of $CaCl_2$ (%)	Polymerization time (min)	Regrowth medium	Recovery (%)	References
Phyllanthus amarus (bahupatra)	Shoot tip	3% NaAlg + MS	6.6	20	MS	90%	Singh et al. (2006)
Physalis peruviana (golden berry)/ nodal segments and shoot tips	Nodal segments, shoot tips	1.5% NaAlg + LS with or without 3% sucrose and 0.5 mg/L ABA	1	20	LS	Max 100% depending on storage conditions for 2 weeks	Yücesan et al. (2015)
Stevia rebaudiana (stevia)	Shoot tips and nodal segments	3% NaAlg + MS	4.4	20	MS + 1 mg/L BAP	100%	Ali et al. (2012)
Citranges [*C. sinensis* (L.) Osb. × *P. trifoliata* (L.) Raf.]	Axillary buds	3% NaAlg + 1/2 R5 medium + 5% sucrose	1.1	15	R5	Max 96%	Chiancone et al. (2016)
Long-lipped serapias (*Serapias vomeracea*)	Protocorm-like body structures	3% NaAlg + Orchimax medium without activated charcoal + 2.0 mg/L ZEA and 1.0 mg/L IBA	3	–	Orchimax + activated charcoal	84%	Bektaş and Sökmen (2016)

NaAlg sodium alginate, *ABA* abscisic acid, *B5* Gamborg medium (Gamborg et al. 1968), *BA* 6-benzyladenine, *GA3* gibberellic acid, *IAA* indole-3-acetic acid, *IBA* indole-3-butyric acid, *KIN* kinetin, *LS* Linsmaier and Skoog's basal medium (Linsmaier and Skoog 1965), *MS medium* Murashige and Skoog's medium (Murashige and Skoog 1962), *NAA* α-naphthalene acetic acid, *PG* phloroglucinol

in a bead-like inotropic gel (calcium alginate) that is called synseed. In most cases, concentrations of these aforesaid solutions and polymerization time must be optimized for the best synseed production. In general, 3% (w/v) sodium alginate and 100 mM calcium chloride for 15–30 min have proved to be the best combination for the formation of an ideal synseed (Tables 1 and 2). Moreover, for an ideal synseed shape, 1000 μL micropipettes are mostly used, and disposable pipet tip was cut from its tip to have a larger opening through which sodium alginate matrix was easily sucked up with a plant tissue. Depending on the sodium alginate matrix composition, encapsulation can be of many forms such as multiwall further coated with similar sodium alginate concentration or can be an irregular shape when the sodium alginate contains full-strength MS medium including calcium chloride ($CaCl_2$) that makes generally amorphous synseed (Gibbs et al. 1999). For better protection, double-layer encapsulation of single-layer synseeds might be applicable; however, it might increase the contamination risks depending on the chemical constituents of the second layer; MS medium thereof presumably increases the infection rates (Sharma et al. 2013).

3.2 Storage, Sowing and Regrowth Patterns of the Synseeds

Synseed technology favors a cost-effective approach for the germplasm conservation ensuring the minimum space for storage and protection from various environmental factors. After an effective storage of the synseeds, cost-effective and disease-free germplasm transportation might be applicable for the plant species; thus, this approach facilitates exchanging germplasm between countries without additional formalities for the quarantine departments at the borders. There are two types of storage used in synseed technology: short- to mid-term storage and long-term storage. In first type of storage, synseed production can be formulated with growth retardants and high osmoticum in polymer matrix, and sometimes concentration of growth medium is reduced to ensure slow-growth conservation after storage at low temperature (ranging between 4 and 14 °C). For the long-term storage, freezing of the tissue might be needed, and encapsulation-dehydration or simple desiccation can also be applicable for longer periods of storage depending on the plant species (Sharma et al. 2013).

In general, increase in storage period of synseeds eventually decreases the regrowth of synseed after sowing. For example, Varshney and Anis (2014) reported a short-term storage for the synseeds of the desert date tree. In their report, considering the cold storage at 4 °C for 4 weeks, the percentage conversion of encapsulated nodal segments into shoots was 82% on growth medium. However, percentage of conversion gradually decreased to 60% after 8 weeks of incubation. Similar pattern was also observed in stevia (*Stevia rebaudiana*) synseeds, and percentage of conversion was reported with a significant decline from 100% at first week of storage to 70% after 3 weeks of incubation at 4 °C in darkness (Yücesan et al. 2015). The basic idea of the long-term storage is based on osmotic and evaporative dehydration of plant cells embedded in high sucrose concentration, which favors the desiccation for

best protection at −196 °C in liquid nitrogen. Since sucrose is an effective and one of cheapest options for the osmoticum that influences osmotic potential in gel matrix, its concentration might be kept at high level by simply applying air-drying method in running laminar flow (Shajahan et al. 2016). After a certain period of storage, synseeds should be placed onto the medium either under axenic or non-axenic conditions for their conversion into the complete plantlets. Regrowth media as shown in Table 2 are variable depending on plant species. Regrowth of the synseeds under in vitro conditions can be achieved using a wide range of medium formulations (i.e., half- or full-strength MS medium with or without growth regulators), and soil/compost mixtures are also applicable in some selected plant species especially for geophytes (Table 2). Once the synseed technology is associated with regrowth under non-axenic conditions, it most likely provides a promising approach for the sowing in an inexpensive way as compared to in vitro techniques. For instance, Saiprasad and Polisetty (2003) reported a successful regrowth of three different orchids (*Dendrobium*, *Oncidium*, and *Cattleya*) in pots containing either charcoal or briquette pieces. Similarly, Bektaş and Sökmen (2016) reported synseed production of *Serapias vomeracea* (an orchid spp.) using protocorm-like body structures. Their results showed that percentage germination of synseed on sterilized peat medium was 60% in pots, while the synseeds lost their viability as a result of fungal contamination in the non-sterilized peats. In the same report, germination rates of synseed from protocorm-like structures were found to be higher than natural seeds derived from zygotic embryos under in vitro conditions. Direct sowing of the synseeds under non-axenic conditions also skips acclimatization procedure required for in vitro systems; thereby, it can be more commercially viable in a cost-effective way of the plantlet production in vitro.

4 Future Prospects

Despite the ample number of publications in plant tissue culture, majority of those publications are mainly focusing on propagation techniques apart from their conservation, storage, and transportation. Over 30 years, synseed technology has attempted to minimize these limitations regarding commercialization of the approach in large-scale production. The success of a propagation protocol cannot always address the success of synseed regrowth, unless certain conditions such as the preference of the best nursery medium, storage temperature, and its duration are determined accordingly. Once the studies on somatic embryo production are associated with synseed production properly, limiting factors as presented in this chapter can be minimized as summarized briefly in Table 3. Apparently, non-embryonic meristematic tissues are of great potential for the synseed production; thus storage and transportation of the germplasm can be cost-effective commercial applications. Chemical constituents of gel matrix and formulations of artificial endosperm associated with synseed technology are also another aspect to be considered recommending germplasm conservation on a commercial scale.

Table 3 Summary of the advantages and limitations of synseed technology

Advantages	Limitations
Sowing synseeds directly in ex vitro conditions is possible	But this is the case for certain species; direct sowing is mainly restricted to geophytes
Plant tissue culture is ideal system for continuous synseed production	But mostly growth medium is needed for the conversion under in vitro conditions
Synseeds are ideal for deliverance	But storage conditions and viability should be checked clearly prior to delivery
Cold storage is possible	Depending on the temperature, duration of storage, and genotype factors, percentage of conversion is supposed to be affected
Unlike the real seeds, artificial endosperm can be modified	Polymerization of artificial endosperm can be shaped by the chemical composition of matrix which might result in an amorphous encapsulation
Double-layer encapsulation may provide extra protection	Labor intensive and costly
Somatic embryo can be effectively produced in vitro	Maturation of somatic embryos and conversion rate are major bottlenecks for synseed
Non-embryonic tissues can be promptly used for synseeds	Due to the unipolar growth development, additional inducers are needed for root formation
Hydrated gels are useful for the encapsulation	More difficult to store, since embryo respires and capsules dry out quickly
Alginate is the best coating material	But still improvement is needed; polymer matrix formulation may affect the shape of the synseeds
Desiccation may increase shelf life of synseeds	But it may damage the embryo in capsules

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Synthetic Seeds: An Alternative Approach for Clonal Propagation to Avoiding the Heterozygosity Problem of Natural Botanical Seeds

Biswajit Ghosh and Sk Moquammel Haque

Abstract The seed is a functional element of sexual reproduction of higher plant. In nature, the humble beginning of the independent life of higher plants starts along with seed germination. Seeds are the "mysterious genetic capsules" which store the genetic information and carry forward to next progeny. The zygotic embryo present inside the botanical seed serves as propagule to produce offspring, and these embryos are always heterozygous because of the recombination during meiotic crossing over in the course of gamete formation as well as for mix-up of the genome of two different parents through cross-pollination. In seed-propagated crops, the agricultural yield is highly unstable due to heterozygosity among seed-derived plants. The answer of this problem is synthetic seeds—the functional mimic of botanical seeds.

Synthetic seed is one of the most promising tools of plant biotechnology, which could be tailor-made for horti- and agricultural improvement at present as well as upcoming days. As all the propagules used for synthetic seed preparation are produced through in vitro clonal propagation, which means they did not encounter two fundamental events of sexual reproduction, the meiotic recombination (during crossing over) and gametic fusion of two different parental genome (cross-pollination), both of these events can create new types of heterozygosity in zygotic seeds. Therefore synthetic seed-derived offspring are always true to type to their source plant. Although, unlike zygotic seed, new types of heterozygosity are never generated in synthetic seeds, the heterozygosity already existed in the mother plant is always transmitted in all synthetic seed-derived offspring.

However, the heterozygosity problem will be totally avoidable, and production of homozygous synthetic seeds is also possible only by using double haploid source plant, because double haploid plants are always truly homozygous. Otherwise synthetic seed technology can only aid to restrict the formation of new types of heterozygosity in offspring, which are abundant in botanical seeds.

B. Ghosh (✉) · S. M. Haque
Plant Biotechnology Laboratory, Post Graduate Department of Botany, Ramakrishna Mission Vivekananda Centenary College, Rahara, Kolkata, India
e-mail: biswajit@rkmvccrahara.org

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1 Introduction

In nature, the humble beginning of the independent life of higher plants (i.e. spermatophytes) starts from seeds. Spermatophyte is the most latest evolutionary embellishment of the plant kingdom and includes gymnosperm and angiosperm. The key character of spermatophyte is that they are seed bearing, unlike Pteridophyta and other cryptogams. It is really tough to overstate the significance of seeds for the evolutionary prosperity of the spermatophytes and the development of human civilization, improvement of human cultures, and their existence (Knapp 2015; Sabelli and Larkins 2015). Plants produce seeds for their most important purpose of life—reproduction. Although gymnosperm and angiosperm both are seed-bearing plants, gymnosperms are more primitive as they have uncovered seeds; in contrast the angiospermic seeds are enclosed within fruits. Plants store enough nutrients within the seed for utilization of their zygotic embryos (Ali and Elozeiri 2017). Seeds can be divided into two groups on the basis of their nutrient storage tissue—albuminous seeds (endosperm tissues serve for storage) and exalbuminous seeds (cotyledons serve for storage). Due to the presence of these nutrient storage tissues, seeds perform as an important element of the world's diet (Bewley 1997).

In higher plants the life cycle is divided into two phases—sporophyte and gametophyte (Haque and Ghosh 2016a). The dominant phase is diploid sporophytic stage where the main plant body occupies maximum span of the life cycle, whereas haploid gametophytic phase (pollen and ovule) is too much reduced and occupies very little span of the life cycle. The gametophytic generation starts from microspore (male gametophyte) or from megaspore (female gametophyte) and ultimately produces sperm and egg cell, respectively (Yadegari and Drews 2004). These haploid sperm and egg cells fertilize together to form a single diploid cell, i.e. zygote—the first cell of the sporophytic generation. Fertilization activates a complex cellular programme that converts two highly specialized haploid germ cells, the sperm and the oocyte, into a totipotent diploid zygote (Clift and Schuh 2013). The first part of the sporophytic development starting from zygote formation up to embryo maturation takes place within the ovule, and ultimately the ovule gives rise to a seed. The initial step in seed development is a double fertilization, where first fertilization occurs between sperm nucleus and egg nucleus to form the diploid zygote, whereas second fertilization occurs among one sperm nucleus and two central cell nuclei, resulting in the development of triploid nutritive endosperm (Sabelli and Larkins 2015). The components of mature seeds—embryo (propagule), endosperm (storage food), and seed coat (protective jacket)—are derived from the fertilized egg cell ($2n$), fertilized central cell ($3n$), and ovule integuments (diploid mother tissue), respectively (Drews and Koltunow 2011). Contemporary genetic studies point out that the

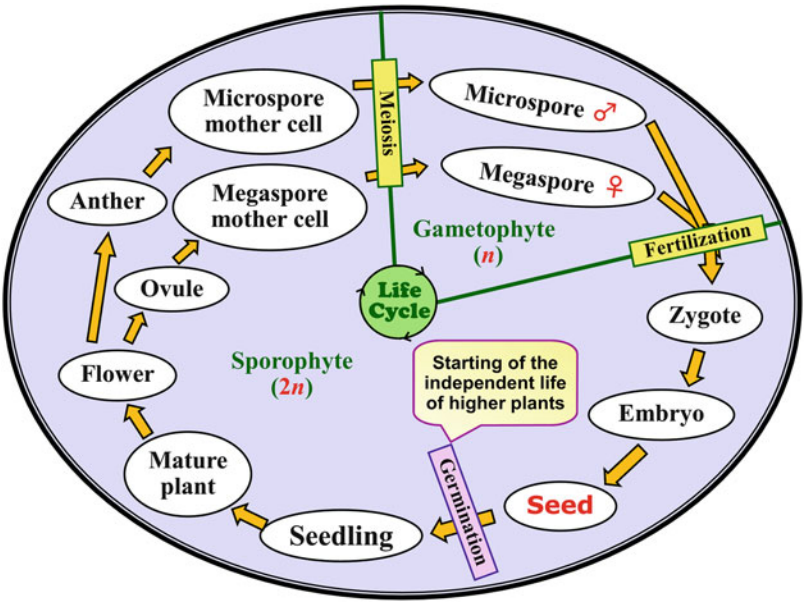

Fig. 1 Diagrammatic representation of the life cycle of seed-bearing plants

female gametophyte influences the events of seed development through maternal-effect genes as well as by regulating maternal contributions (Yadegari and Drews 2004; Drews and Koltunow 2011). The development of the seeds takes place in the mother plant; as a result zygote to embryo formation and their maturation are fully dependent on mother plants (Maheshwari 1950). Therefore, the independent life of higher plants eventually starts from seed germination, and the ultimate aim of their life is to produce seeds for the next generation (Fig. 1). Starting from zygote, the sporophytic plant bodies develop via embryo development, different phases of embryo maturation, embryo germination, and the vegetative growth (roots, stems, leaves) of the main plant body. After prolonged vegetative developmental phase, they attain certain maturity, and then transition from vegetative to flowering stage occurs (Haque et al. 2018). Female and male reproductive organs develop within the flowers, and ultimately haploid spores (mega- and microspores) are produced through reductive (i.e. meiosis) cell division (Haque and Ghosh 2017a).

"Evolution is the process of heritable change in populations of organisms over multiple generations" (https://www.nature.com/subjects/evolution). Evolution in living organisms is a very essential process which creates a gradual modification of all forms of life over generations. Without evolution, all life gets threatened and ultimately goes to extinction in ongoing changing environments of the earth (Burger and Lynch 1995; Parmesan 2006). The four major factors which lead to the evolution process are mutation, gene flow, genetic drift, and natural selection (Allendorf 2017). Evolution occurs in populations because of the modifications in allele frequency over time. Modification of allele frequency increases the heterozygosity

in individuals as well as in populations. Hence, more heterozygosity means more probabilities of speciation, i.e. formation of new species. This marvel of heterozygosity is utilized by the plant breeder as a weapon for creating new hybrid varieties (Acquaah 2012). At this point, heterozygosity serves as blessing for the progress of agriculture. But whenever a hybrid line was created, maintenance of genetic stability of this hybrid line is very essential, which is only possible through clonal propagation of this hybrid plant (Wang et al. 2019). Clonal propagation is none other than a vegetative mode of propagation, where all the offspring truly maintain the genetic make-up of their parent plant by avoiding the genetic recombination, i.e. meiotic crossing over. Some of the organisms have ostensibly evolved without sexual reproduction for several centuries (Schön and Martens 2003). Due to human-exercised selective pressures, the clonally multiplied food crops incorporate an enormous range of ecological, morphological, and phylogenetic diversity (McKey et al. 2010). Somatic mutations are the cause of genetic variation among clonally propagated domesticated crop plants which supports the adaptive evolution (Whitham and Slobodchikoff 1981).

Seeds are the functional component of plant reproduction, from where the independent life of higher plants starts. In general, the botanical seeds comprise three basic criteria—(1) first and foremost it contains a propagule in the form of zygotic embryo, (2) the zygotic embryo is covered by a hard jacket, i.e. seed coat for mechanical protection, and (3) it contains storage food for zygotic embryo in the form of nutritive tissue, i.e. endosperm or cotyledon (DuPont 2012). Apart from these three basic criteria, though not all, most of the seeds have another important features—seed dormancy (Yildiz et al. 2017). During dormancy period the seeds cannot germinate even in the presence of favourable environmental conditions (temperature, humidity, oxygen, and light) required for germination. Seed dormancy is a trait acquired by the spermatophytes during evolution to subsist in adverse environments such as low or high temperature, salinity, and drought (Yildiz et al. 2017). Dormancy can compare with “sleep” and dormancy-break with “wake-up”. After dormancy-breaking the seed germinates and give rise to a seedling. However, dormancy-break is very unpredictable because the threshold stimulus needed to encourage germination differs extensively among individual seeds; therefore all seeds among the same population do not germinate synchronously (Bewley 1997). Seed germination frequency is considered as a determining factor for plant productivity (Ali and Elozeiri 2017). Although seed propagation is the leading mode of reproduction of higher plants, there are some drawbacks like all seedlings are genetically not true to type, production of seed is not possible throughout the year but restricted to a particular season only, and few species aren’t able to produce seeds throughout their life. Hence, an alternative of botanical seed is strongly desiderated to address the above-mentioned drawbacks.

Synthetic seeds are functionally alternatives of botanical seeds and are tailor-made and developed in laboratories. Synthetic seed is one of the most promising plant biotechnological tools which could be expedient for agricultural improvement at present as well as upcoming days (Haque and Ghosh 2014). This technology has been established to utilize somatic embryos or some other micropropagules like

shoot tips, nodes, etc. as seed analogues effectively in the greenhouse or field and their commercial planting (Ara et al. 2000). Nowadays, the synthetic seed is an ardent topic of research, and importance of this technology can be predicted by the huge number of scientific works continuously done on it. A casual perusal of the scientific search engines (https://www.sciencedirect.com/) reveals that over 4450 publications related to "synthetic seed or artificial seed" have been published in the last 3 years (2017–2019, accessed on February 4, 2019). In crop plants, the maintenance of genetic stability of the high-yielding variety and retain of the high-yielding features in next generation is very essential, which is possible only through clonal propagation (Bhojwani and Razdan 1996). Since all zygotic seeds are heterozygous (except when parents are inbred line), therefore seed-derived plants are genetically not true to type to their parents; henceforth, all the desired characters of the parents may not be expressed in offspring. Heterozygosity is a realistic problem for those crop species, whose planting material is zygotic or botanical seeds. Over botanical seeds, the synthetic seeds have some advantages like synthetic seed-derived seedlings are true clones of their source plant, and it can be produced in huge quantity throughout the years (Bapat and Mhatre 2005).

2 Propagation Through Seeds

Seed propagation is the method of plant reproduction through seeds. Maximum plant species naturally reproduces through seed propagation. As well, farmers also take advantage of seed propagation for cultivation of agricultural and horticultural crops. Seeds are an essential element of the life cycle of higher plants, as they store the hereditary information essential for the next progeny to disperse, inaugurate, grow, and finally reproduce to perpetuate the species (Nambara and Nonogaki 2012). Seed formation in higher plants begins along with the developmental decision to switchover from a vegetative to a reproductive phase of development (Simpson et al. 1999). The seeds contain a lot of secrets that have yet to be discovered, that's why Nambara and Nonogaki (2012) mentioned seed as "mysterious genetic capsules". In this type of plant propagation, seeds can be germinated, post-germination development occurs, and ultimately a seedling was developed. In recent years, an understanding of the seed biology especially seed dormancy and germination has been greatly progressed (Nonogaki 2017). In nature, germination postponement due to dormancy keeps certain seedlings safe from possible damage of detrimental weather or from seasonally migrating herbivores (DuPont 2012). Seed germination is a very crucial event in agricultural aspects, and yield may directly depend on the percentage of seed germination. There are four environmental factors which affect germination—water, oxygen, light, and temperature; and germination is rapid and uniform at optimal temperature and moisture (DuPont 2012). Seed vigour is a most important agronomic trait determined by longevity during storage, germination capability, and growth of the seedling in field condition (Daniel 2017). Two types of seeds are found in nature, albuminous seeds and exalbuminous seeds (Fig. 2).

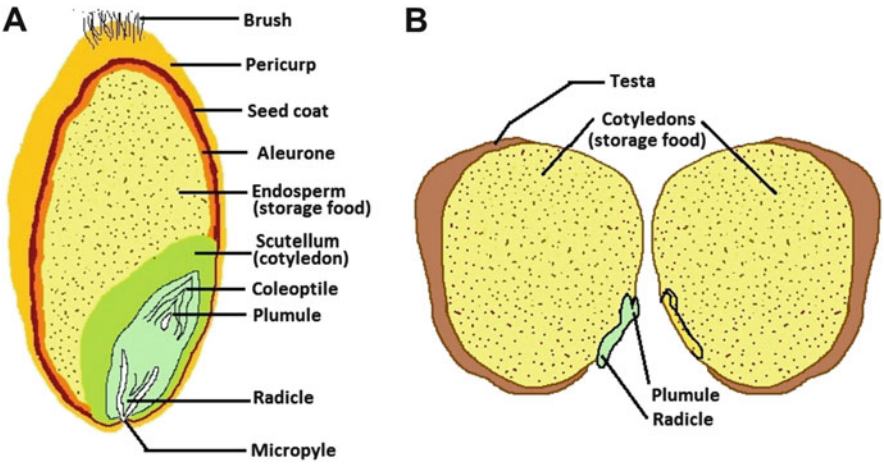

Fig. 2 Diagrammatic representation of (**a**) albuminous seed (wheat) and (**b**) exalbuminous seeds (chickpea)

Albuminous seeds have endosperm, a special nutritious tissue for food storage, which remains persistent even at maturity (e.g. rice, wheat, castor seed, etc.). Here cotyledons merely performed as nutrient-sucking organs. In contrast, the exalbuminous seeds are those seeds where endosperm is used up by the developing embryo and cotyledons turn thick and fleshy and serve as food storage tissue (e.g. *Alisma plantago* seed, chickpea, jackfruit seed, etc.). In dicotyledonous albuminous seeds, the endosperm is solely made with uniform living reserve cells, whereas, in monocotyledonous albuminous seeds, the starchy endosperm mostly consists of nonliving storage tissue, enclosed by the living aleurone layer (Joët et al. 2009).

3 Propagation Through Isolated Zygotic Embryo Culture

Zygote is the progenitor of subsequent generation, which forms an embryo through sequential developmental stages (Bhojwani and Dantu 2013a). Zygotic embryo culture refers to an aseptic excision of the zygotic embryo (generally in immature conditions but sometimes at mature stage) from seeds and their in vitro culture in artificial nutritive medium (in absence of endosperm tissue) with aim to obtaining complete plants. Excision of embryos from seeds and their in vitro culture were first time initiated by Hannig in more than 115 years ago (Hannig 1904). Three main utilities of zygotic embryo culture are:

1. A rare hybrid can be obtained through immature zygotic embryo rescue and their culture, because in reciprocal cross among two distantly related species (during

interspecific and intergeneric hybridization), the fertilization occurs but endosperm tissue is not developed or degenerated; as a result further maturation of embryo hampers due to lack of nutrition, and ultimately embryo is aborted if not rescued (Sahijram et al. 2013). Nowadays it is potential to rescue hybrid zygotic embryos which are mostly aborted even at the early stage of development, i.e. globular stage, and the method of hybrid zygotic embryo culture has come to be an important part of the plant breeding methodology (Bhojwani and Dantu 2013a; Wang et al. 2019). In vitro zygotic embryo culture is now very popular and being regularly used to produce rare hybrids which may be possibly not produced by the conventional breeding method because of sexual incompatibility between the male and female parents at postfertilization stages (Rajamony et al. 2006; Eeckhaut et al. 2007; Sahijram et al. 2013; Gupta et al. 2019).

2. The zygotic embryo culture is also exploited for overcoming the dormancy of recalcitrant seeds (Raghavan 2003). In case of coat-enhanced seed dormancy, the embryos excised from these seeds remain not dormant (Bewley 1997). Therefore, the excised embryo of coat-enhanced dormant seed when cultured in vitro on nutrient medium can grow by breaking the dormancy.
3. In addition, zygotic embryo-derived callus possesses a high regenerative capacity as compared to mature organ (leaf, stem, root)-derived callus; hence zygotic embryo is a good source of explant for developing callus-mediated indirect organogenesis or embryogenesis and plant regeneration. There are so many micropropagation protocols that had been established in gymnosperms and angiosperms where zygotic embryos are used as initial explants (Fitch and Manshardt 1990; Bodhipadma and Leung 2002; Chaturvedi et al. 2004; Zhang et al. 2006; Yang et al. 2008; Konieczny et al. 2010).

4 Heterozygosity in Seeds

Zygosity is the degree of resemblance among the genetic alleles for a particular trait in an organism. While some traits exhibit the occurrence of just a single allele, lots of others show the existence of two or more alleles for a particular locus within a population. In diploid plants, one allele is inherited typically from the female parent and another from the male parent. On the basis of similarity or dissimilarity of DNA sequence among these homologous alleles, the genetic trait is considered to be homozygous or heterozygous, respectively (Fernandez 2013) (Fig. 3). Hence, heterozygosity is the form of having two dissimilar alleles at a locus, and it is fundamental for studying genetic variation within populations. Mutation, natural selection, genetic drift, and migration play critical roles on maintaining heterozygosity in populations (Allendorf 2017). During meiosis, crossing over among two non-sister chromatids of homologous pair resulted reciprocal exchange of genetic materials, which ultimately mixed up the hereditary factors of male and female parents and transgresses in offspring (Clift and Schuh 2013). Hence, sexual reproduction especially crossing over plays a crucial role on creating as well as

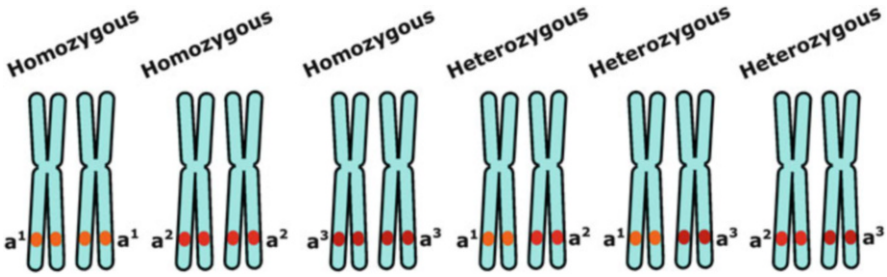

Fig. 3 Graphical representation of homologous chromosome showing heterozygosity and homozygosity concept. If three different alleles (a^1, a^2, a^3) of the same gene are present in a population, then three types of homozygous (a^1a^1, a^2a^2, a^3a^3) and three types of heterozygous (a^1a^2, a^1a^3, a^2a^3) individuals may present within this population

maintaining the heterozygosity. Therefore, all the botanical seeds containing zygotic embryo, which are produced by the random fusion of two meiotic products, i.e. sperm and egg cell, are always heterozygous in nature.

4.1 Heterozygosity and Plant Breeding

During Mendel's time, people have no idea regarding the genetics, but farmers realized that plants may perhaps be changed vividly through cautious selective breeding. The resilient, strong, disease-resistant wild relatives of crop plants were crucial for cross-breeding programme (Acquaah 2012). Since then, simultaneously with natural cross-breeding, the human being was also trialled with various types of cross-breeding to obtain high-yielding, disease- and drought-resistant hybrid plants which are better for cultivation. Mendel's famous experiment on *Pisum* revealed segregation of traits, established the function of gametes as the carriers of genetic factors, and established the mutual significances of segregation and recombination (Pupilli and Barcaccia 2012). Heterozygosity, genetic diversity, natural selection, and mutation, all of these may lead speciation and hence evolution. For example, an only ancestor species of weedy coastal mustard in due course of evolution gave rise to over half dozen of accustomed European vegetables (Hanson 2013). Hence, heterozygosity is one of the main causes of genetic diversity and speciation (Avise 1977; Allendorf 2017).

Plant breeding is a mechanism for the improvement of plants by hybridization or selective mating for the benefit of human beings. Traditionally it serves as tool for the production of new plant varieties for upliftment of agriculture and horticulture (https://www.nature.com/subjects/plant-breeding, accessed on February 4, 2019). In plant breeding the "inbred" line is those plants where every single locus is homozygous. Generally inbred lines are produced by repeated self-pollination followed by selection for minimum 6–10 consecutive generations to attain the almost homozygous condition (Prigge et al. 2012). Nowadays, apart from the conventional breeding

method, another popular and time-saving in vitro biotechnological method is available for truly homozygous line production—i.e. double haploid plant production (Ren et al. 2017; Kleter et al. 2019). These truly homozygous or inbred lines are very essential in a plant breeding programme for hybrid production (Dong et al. 2019). Plant breeding has given rise to many new varieties of seed crop with high levels of carbohydrates, proteins, fats, or certain combination of those three in their seeds (Krebbers et al. 1997).

4.2 Demerits of Heterozygosity

In today's agriculture, hybrid crops are very important for their high-yielding potentiality, but required lucrative phenotypes are lost in the progeny of subsequent generations due to genetic segregation (Wang et al. 2019). Once the hybrid line is produced, most of the time we want exact true-to-type offspring, which permits the continuation of the desired phenotypic characteristics of the hybrid cultivar and helps to maintain the stable high yield. When the requirement is true-to-type plant production, then the above-mentioned (see Sect. 4.1) merits of heterozygosity are considered as demerits. Zygotic embryo present inside the botanical seed serves as a natural propagule to produce offspring, and these propagules are always heterozygous because of the crossing over during meiosis as well as cross-pollination among two different parents. Phenotypic expression from heterozygotes failed to maintain same agronomic quality compare to that of source plant (Küpper et al. 2010). Now, when the target is to produce true-to-type parental plants, sexual reproduction creates problems and does not provide desired characters at that time; alternatively clonal propagation is appropriate to serve the purpose (Wang et al. 2019).

4.2.1 Heterozygosity Formation Due to Crossing Over in Meiosis-I

Meiosis, the exclusive and essential event of the life cycle of the entire range of sexually reproducing organisms (Wijnker and Schnittger 2013), is the procedure through which a diploid sporophytic cell gives rise to haploid spore cells which grow further to develop the gametophyte and ultimately the gametes (Schwarzacher 2003). The first meiotic division (i.e. meiosis-I) is very crucial for sexually propagated plant species because of the two major events. First is the reduction of the chromosome number to half of their somatic number to produce haploid gamete; therefore the meiotic division is alternatively known as reduction division. During fertilization the gametic fusion of microspore and megaspore (i.e. ♂ and ♀ gametes) gives a diploid zygote. Thus reduction division is the only way to maintaining the chromosome number characteristic of the sexually reproduced species. Second is recombination in crossing over that takes place during the pachytene stage of first meiotic prophase, where the hereditary factors from male and female parents get mixed due to the reciprocal exchange among non-sister chromatids (Schwarzacher

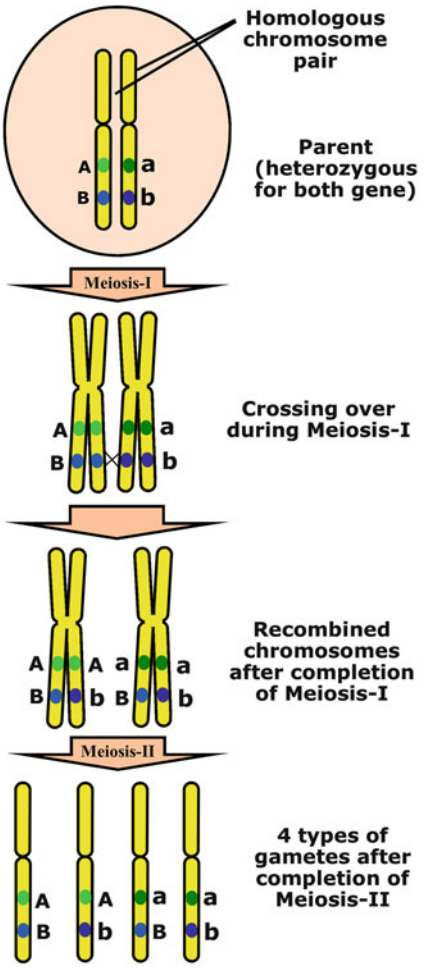

Fig. 4 Diagrammatic presentation about how meiotic crossing over creates new recombinant types in gametes. Present example considers only one pair of homologous chromosomes and only two genes in two loci, and both genes have only two alleles, but in nature, several pairs of homologous chromosomes are present in each individual, and few thousands of genes reside in different chromosomes as well as multiple alleles of each gene are present in population which remain more complex and create diverse types of heterozygous gametes

2003). This type of mix-up of genetic materials causes rearrangement of alleles and enhances the probability of heterozygosity, and segregation may arise among the progeny (Zhang et al. 2019). For example, if we consider only two genes and both have only two alleles (A, a, and B, b) and crossing over takes place in one locus and other genes have not participated in crossing over, then after meiosis four different types of gametes (AB, Ab, aB, ab) will be produced (Fig. 4). If crossing over does not happen, then only two types of gametes (AB, ab) will be produced. Hence, crossing over increases the recombinant types in gamete and therefore increases the chances of heterozygosity in offspring. However, the present example (Fig. 4) considers only one pair of homologous chromosomes, only two genes in two loci, and both genes having only two alleles, but in nature, more chromosomes are present in every individual, and a huge number of genes (few thousands) reside in different chromosomes, and, most of the time, multiple alleles of each gene are present in

population which obviously creates more complexity and generates several new types of heterozygous gametes.

4.2.2 Heterozygosity Formation Due to Random Cross-Pollination

In the previous section (see Sect. 4.2.1), it was already discussed about the role of meiotic crossing over on creation of new types of heterozygosity. Now, even if we don't consider the crossing over phenomenon (i.e. let's assume that the crossing over did not happen), all offspring are not always true to type because of the random cross-pollination. Specifically, when pollen and ovule are from different parents, their genetic make-up is also different in nature (Acquaah 2012). Therefore, all zygotes produced in a particular plant may not be genetically identical because though the female recipient germ cell was fixed, the male donor (i.e. source of pollen grains) is different. For example, consider only two genes and both have four alleles (A^1, A^2, A^3, A^4 and B^1, B^2, B^3, B^4), male parent with $A^1A^2B^1B^2$ and female with $A^3A^4B^3B^4$ (Fig. 5). Let's assume that the crossing over did not happen and then after meiosis two dissimilar types of gametes (A^1B^1, A^2B^2) are produced in male parent and two additional types of gametes (A^3B^3, A^4B^4) are produced in female parent. As a result of random cross-pollination, four totally new types of heterozygosity are produced among offspring ($A^1A^3B^1B^3$, $A^1A^4B^1B^4$, $A^2A^3B^2B^3$, $A^2A^4B^2B^4$), and none of them are similar with any parent ($A^1A^2B^1B^2$, and $A^3A^4B^3B^4$). However, in the present example (Fig. 5), it considered only one pair of homologous chromosome, two genes in two loci, and both genes having four alleles (two alleles in each parent), but in nature, several pairs of homologous chromosomes are present in each individual, and few thousands of genes reside in different chromosomes as well as multiple alleles of each gene are present in population which remain more complex and create diverse types of heterozygosity. The next-generation sequencing technique provides the facility to screen 10–100 of thousands of loci all over the genome for detecting heterozygosity, which has reformed our understanding of heterozygosity in natural populations (Allendorf 2017). Hence random cross-pollination also causes mix-up of different alleles present in the population and also enhances the probability of new form of heterozygosity in offspring (Acquaah 2012).

5 Clonal Propagation and Its Importance

Multiplication by means of non-sexual mode of propagation when all the multiplied copies are genetically identical to their parent is called clonal propagation. Plant population produced from a particular individual plant through non-sexual mode of propagation creates a clone. All clones are genetically true to type to their source plant. In natural condition, clonal plant propagation occurs by vegetative propagation or by apomixis (Park et al. 2016). Several plant species that propagate clonally (non-sexually) are also capable of sexual reproduction (Bailey 2018). Clonal

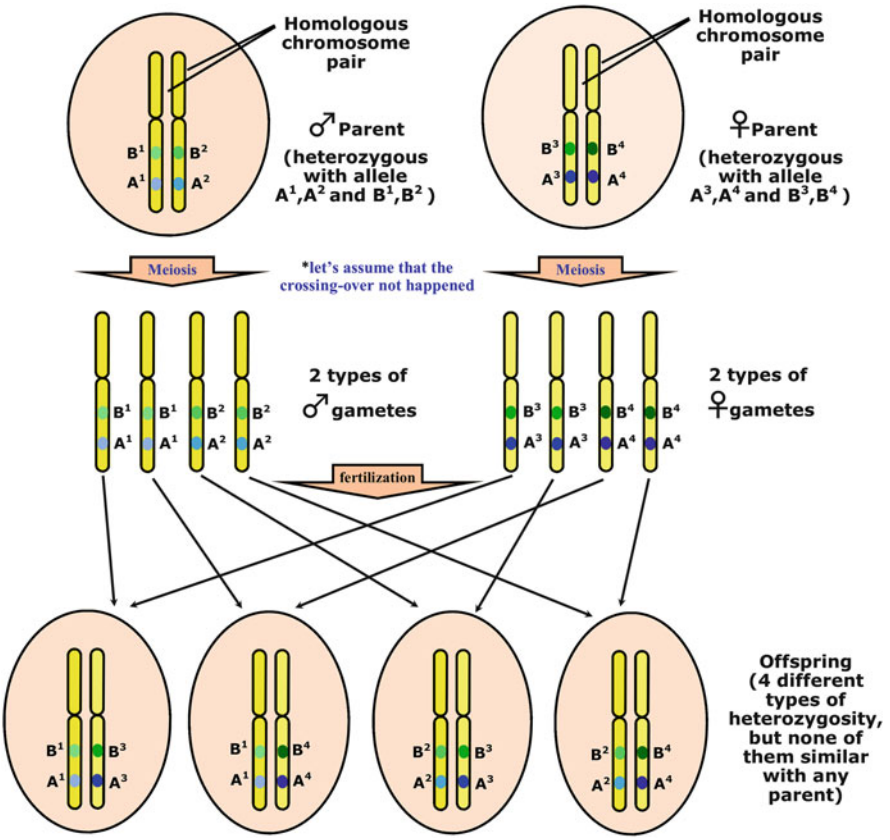

Fig. 5 Diagrammatic presentation about how cross-pollination enhances the probability of heterozygosity in offspring. Four totally new types of heterozygosity are produced among offspring ($A^1A^3B^1B^3$, $A^1A^4B^1B^4$, $A^2A^3B^2B^3$, $A^2A^4B^2B^4$), and none of them are similar with any parent ($A^1A^2B^1B^2$, and '$A^3A^4B^3B^4$). Present example considers only one pair of homologous chromosome and two genes in two loci, and both genes have four alleles (two alleles in each parent), but in nature, several pairs of homologous chromosomes are present in each individual, and few thousands of genes reside in different chromosomes as well as multiple alleles of each gene are present in a population which remains more complex and creates diverse types of heterozygosity

propagation has some advantages over sexual mode of reproduction—fixation of valuable agronomical traits, control of gene flow from wild-to-crop plant, and easiest way of multiplication (Bhojwani and Razdan 1996). As well, the clonal propagation also has some drawbacks like restriction on genetic diversity, deleterious mutations, retain of pathogenic entities and transfer to subsequent progeny (McKey et al. 2010).

Variability arising between seed-derived plants can be omitted by avoiding sexual reproduction and following vegetative mode of propagation. Unwanted gene flow from wild-to-crop plant can be controlled through clonal propagation (McKey et al.

2010). Clonal propagation is very useful when exact true-to-type plants are required. In this type of propagation, all offspring are genetically identical to their parent, so all the important characters of parental plant are truly unchanged among all progeny. Clonal propagation is the easiest way of multiplication and highly practical in the case of tree propagation, particularly in fruit cultivation; because a seed-derived tree needs several years to reach maturity, whereas a vegetatively propagated (through grafting or gootee) tree requires comparatively much less time to reach the fruiting stage (Bonga 1982; Park et al. 2016). Clones are very useful in the field of agriculture on the way of maintaining the steady production (Bhojwani and Razdan 1996).

6 Different Modes of Clonal Propagation

In higher plants, mainly two types of clonal propagations are observed in nature, viz., vegetative propagation and apomixes (Fig. 6). Besides the natural mode of propagation, human beings too have established a number of methods for artificial vegetative propagation of numerous valuable plant species (Megersa 2017). Again, natural propagation always happens in in vivo condition, whereas man-made artificial methods of propagation through vegetative mode are either in vivo or in vitro condition.

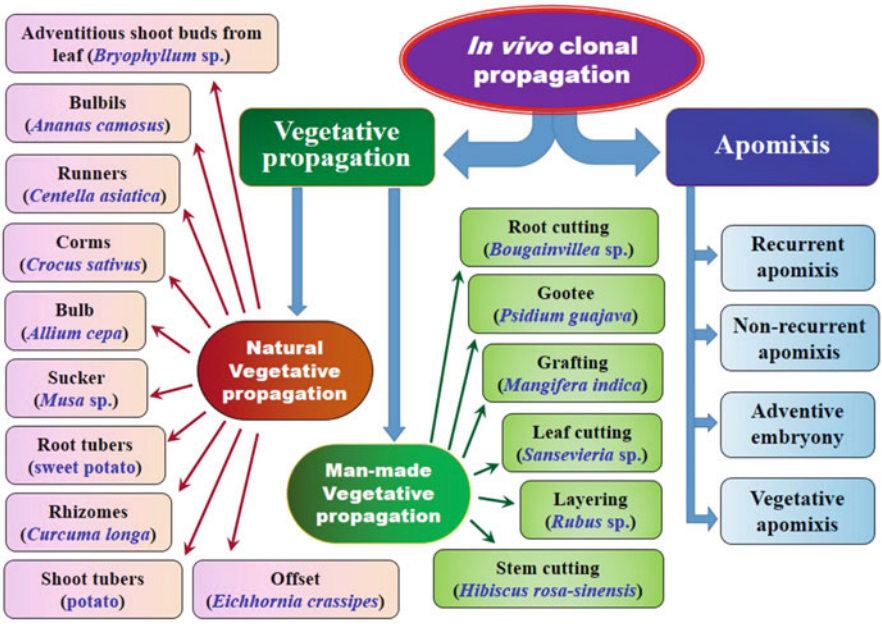

Fig. 6 Schematic representation of different paths of in vivo clonal propagations

6.1 Vegetative Propagation

The vegetative mode of propagation is very common and adopted by numerous plant species, even though many of them are also capable of sexual reproduction (Bailey 2018). Different plant organs like root, stem, and leaves are modified in a different way for vegetative propagation (Megersa 2017). Few of the very common structures which are modified for vegetative reproductions are root tubers (sweet potato), adventitious shoot buds from root (*Albizia lebbeck*, *Aegle marmelos*), bulbs (onion, *Ledebouria revoluta*, *Drimiopsis botryoides*), rhizomes (ginger, *Curcuma longa*, *Alpinia calcarata*, *Kaempferia angustifolia*), tubers (potato, *Oxalis tuberosa*), runners (strawberry, *Centella asiatica*), offset (*Eichhornia crassipes*), corms (*Crocus sativus*, *Gladiolus*), bulbils (*Ananas comosus*), sucker (*Musa* spp.), adventitious shoot buds from leaf (*Bryophyllum* spp.), etc.

In higher plants, where natural way of vegetative reproduction is absent, few special methods had been developed by humans for clonal propagation. These methods are mainly applied for the propagation of horticultural as well as fruit plants (Bailey 2018). Some of these man-made methods are as follows—Cutting is a method where a plant part, usually a stem or root or leaf, is separated from mother plants and sometimes treated with plant hormones and then planted in moist soil. Adventitious organs are developed from the cuttings, and ultimately a new plant was produced. Rose, *Hibiscus rosa-sinensis*, and *Chrysanthemums* spp. are some common examples of stem cutting; similarly *Bougainvillea* spp. are propagated through root cutting and *Sansevieria* sp. through leaf cutting. Grafting is another method where a shoot tip (or scion) was collected of the desired source plant and grafted on the stem of another healthy seed-derived plant (or stock). In due course, the tissue of stock and scion become attached together to form a complete plant (e.g. Mango, *Adenium*). Few other popular methods are layering (*Rubus* sp.), gootee (*Psidium guajava*, *Citrus*), etc. (Bailey 2018).

6.2 Apomixis and Clonal Seed Propagation

Apomixis refers to a process of non-sexual propagation through seeds, in absence of the meiotic cell division as well as gametic fusion, producing clonal offspring of maternal origin (Spillane et al. 2004). In 1908 Winkler for the first time coined the term "apomixis" to mean "substitution of sexual reproduction by an asexual multiplication process without nucleus and cell fusion" (Winkler 1908). Since this is a fertilization-independent, spontaneous natural development of the embryo from somatic cell ($2n$) without any gametic fusion, so perhaps it can be said in other words as "natural somatic embryo". In agriculture the apomixis is employed as a reproductive tactic for clonal plant production by seeds (Spillane et al. 2001; Bicknell and Koltunow 2004; Pupilli and Barcaccia 2012). The plants produced through apomixis are known as apomictic plant, which is not very uncommon

among higher plants, and more than 400 species of about 40 families are apomictic (Bicknell and Koltunow 2004). In higher plants apomixis is defined as the asexual development of a seed from the female parental tissues ($2n$) of the ovule, bypassing two utmost fundamental events of sexual reproduction—meiosis and fertilization, leading to the development of an embryo. The momentary definition of apomixis defines an end product, but the developmental procedures that lead to this end result can differ broadly (Ozias-Akins 2006). Primarily four kinds of apomixis are found in nature:

1. Recurrent apomixis: here an embryo sac develops from the megaspore mother cell where meiosis has not happened or from some adjoining cell; therefore, the egg cell is diploid. An embryo develops directly from the diploid egg cell ($2n$) escaping fertilization. Some examples of recurrent apomixis are somatic apospory, diploid apogamy, and diploid parthenogenesis.
2. Nonrecurrent apomixis: here an embryo develops directly from a typical haploid egg cell (n) without fertilization; as a result the embryos will also be haploid. Nonrecurrent types of apomixis are rarely found in nature. Particular examples of such types of apomixis are haploid parthenogenesis, haploid apogamy, and androgamy.
3. Adventive embryony: here embryos were developed from cells of nucellus or integuments, outside the embryo sac. In addition to such adventive embryos, the regular zygotic embryo may also develop concurrently within the embryo sac, thus generating polyembryony situation, frequently found in *Citrus* spp.
4. Vegetative apomixis: here instead of flowers, some vegetative buds or bulbils are produced in the axil of inflorescence, and they can be regenerated without any struggle. Such types of apomixis are observed in *Agave*, *Poa bulbosa*, and some grass species.

Apomixis is a beautiful attribute for the improvement of crop cultivation because it facilitates the formation of huge genetically identical populations and maintains hybrid vigour through continual seed production (Spillane et al. 2001; Hand and Koltunow 2014). In plant breeding the apomixis prospered several advantages. During sexual reproduction, cross- and self-fertilization followed by segregation have a tendency to modify the genetic configuration of offspring. Inbreeding and abandoned outbreeding as well have a tendency to interrupt heterozygote superiority in such offspring. In contrast, apomicts have a tendency to protect the genetic configuration as such (Spillane et al. 2001). Apomicts are also proficient of conserving benefits of heterozygote generation after generation. Thus, apomixis offers remarkable advantage in plant breeding where genetic consistency maintained over several generations for both heterozygosity (in hybrids of both outbreeders and selfbreeders) and homozygosity (in selfbreeders) is the remarkable motive (Hand and Koltunow 2014). Furthermore, apomixis may also offer an effective utilization of maternally inherited factors, if present, reflecting in the subsequent offspring.

It is mostly believed that zygotic embryogenesis (sexual reproduction) and apomictic embryogenesis (asexual reproduction) both follow alike developmental

pathways in the course of embryo and seed development (Pupilli and Barcaccia 2012). But the offspring of an apomictic plant are always genetically uniform to their mother plant.

6.3 *In Vitro Clonal Propagation*

Nowadays, in vitro plant cell and tissue culture method is considered as one of the basic components of modern plant biotechnology (Neumann et al. 2009). In vitro methods of plant propagation always follow the vegetative mode of regeneration. Plant growth and development occur in in vitro aseptic and controlled environmental condition in the presence of artificial nutrient medium and plant growth regulators (Jha and Ghosh 2016). The prodigious advantages of in vitro aseptic technique of clonal propagation (i.e. micropropagation) are that an enormous number of disease-free true to type plantlets can be produced within little space and short time span plus season-independent round-the-year production (Bhojwani and Razdan 1996; Altman and Loberant 1998; Anis and Ahmad 2016). Theoretically all living plant cells are "totipotent" and have the capability to produce a whole plant from any single cell. In vitro culture technique is obviously the best platform to utilize the cellular totipotency of the plant cell for clonal propagation (Bhojwani and Dantu 2013b). The most significant and unique capacity of in vitro culture system is—irrespective of the nature of the explant source (root, leaf, shoot tip, node, internode, flower parts, pollen, ovule, zygotic embryo, endosperm, etc.)—a complete plant can be produced via axillary or via adventitious regeneration through organogenesis or embryogenesis (Fig. 7). In axillary regeneration methods, shoot tips and nodes are used as explant, and plant growth regulators (especially cytokinins) are used for inducing axillary branching by breaking the dormancy of shoot buds which are already present in their axil. Profuse branching is induced through this process, and complete plantlets are produced from these multiplied shoots followed by root organogenesis. The adventitious regeneration refers induction of plant organs or embryos from unnatural position, i.e. from where they are not grown in in vivo natural conditions. Plants can adventitiously propagate through two different primary morphogenic pathways, i.e. either through organogenesis (unipolar organs are formed) or through somatic embryogenesis (bipolar embryos are formed). Again, both organogenesis and embryogenesis may go through either direct morphogenic pathway without any callus phase or through indirect morphogenic pathway via callus phase (Fig. 8). The callus-mediated path has been accompanying with an augmented risk of genetic instability and henceforth increasing somaclonal variations among regenerated plants (Hervé et al. 2016). Therefore, the plants produced through direct morphogenic pathway are more reliable when target is clonal propagation, because comparatively more somaclonal variants are induced in callus-mediated regeneration (Bhojwani and Dantu 2013c).

The in vitro somatic embryogenesis process has been routinely used as large-scale micropropagation method (Ghosh and Sen 1989, 1991, 1996; Haque and Ghosh 2016b; El-Esawi 2016). Somatic embryogenesis is a typical example of

Fig. 7 In vitro clonal propagations of different plants through different paths. (**a**–**c**) Multiple shoot inductions from node culture of *Bacopa chamaedryoides*, *Hemidesmus indicus*, and *Physalis minima*; (**d**) multiple shoot inductions from shoot tip culture of *Kaempferia angustifolia*; (**e**, **g**, **h**) adventitious shoot induction from leaf explant via direct shoot organogenesis in *Solanum americanum*, *Bacopa monnieri*, and *Tylophora indica*; (**f**) direct shoot organogenesis from internode explant in *Bacopa chamaedryoides*; (**i**) direct somatic embryogenesis from leaf explant in *Ledebouria revoluta*; (**j**, **k**) indirect somatic embryogenesis via callus phase in *Ledebouria revoluta* and *Drimiopsis botryoides*; and (**i**) indirect shoot organogenesis via callus phase in *Tylophora indica*

cellular totipotency concept which is expressed in a huge number of plant species (Verdeil et al. 2007; Loyola-Vargas and Ochoa-Alejo 2016). All living plant cells cannot be deliberated as totipotent per se, but few of them can reclaim totipotency in

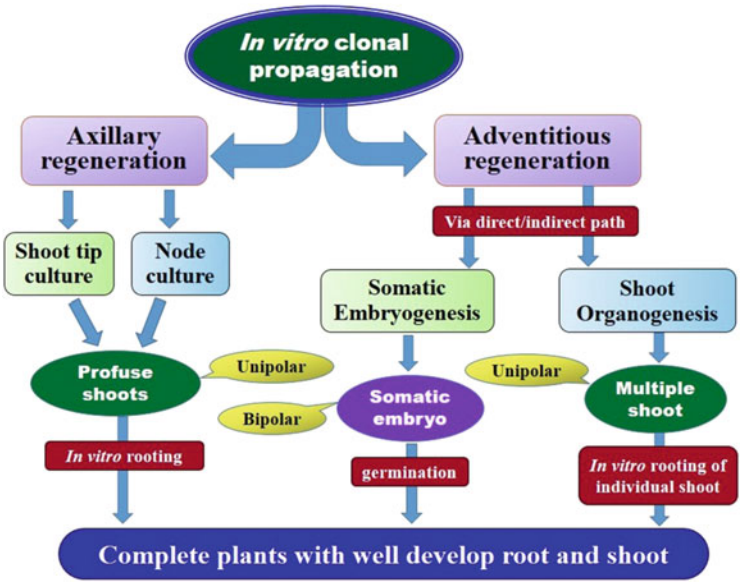

Fig. 8 Schematic representation of different paths of in vitro clonal propagations

suitable environments (Fehér et al. 2016). Somatic embryogenesis is considered to be developmental reprogramming of somatic cells or non-sexual cells towards the embryonic pathway followed by development through typical morphological stages (Yang and Zhang 2010) that are similar to the zygotic embryo development (Leljak-Levanic' et al. 2015). In somatic embryogenesis process, the somatic cell is distracted from their usual fate and reprogrammed an entire ontogenic developmental process to form embryos without any gametic fusion or zygote formation. Somatic and zygotic embryogenesis represent similar developmental events in which single cells obtain embryogenic cell fate and redifferentiate into mature embryos (Harada et al. 2010). For evidence, the developmental study between zygotic and somatic embryos of oak (*Quercus robur*) exhibited nearly four to seven identical developmental stages among them (Palada-Nicolau and Hausman 2001). During fertilization, two haploid (n) gametes fuse together to form a diploid ($2n$) zygote. The zygote is truly a totipotent single cell, from where an embryo is formed by way of gradual differentiation process. The embryo produced from a single zygotic cell is known as zygotic embryo, which is the propagule present inside the botanical seeds. Hence, fertilization is a must-needed process on the way of zygotic embryo production. The zygotic embryo is a bipolar structure having an embryonic axis and cotyledons. Monocotyledonous embryos have single cotyledon, while dicotyledonous embryos have double. The embryonic axis contains radicle (root initial) and plumule (shoot initial) at their two ends. In the course of somatic embryogenesis, the fertilization did not happen; instead, the embryo is developed directly from diploid ($2n$) somatic cell without fusion of two haploid (n) gametes. Therefore, the somatic embryogenesis is

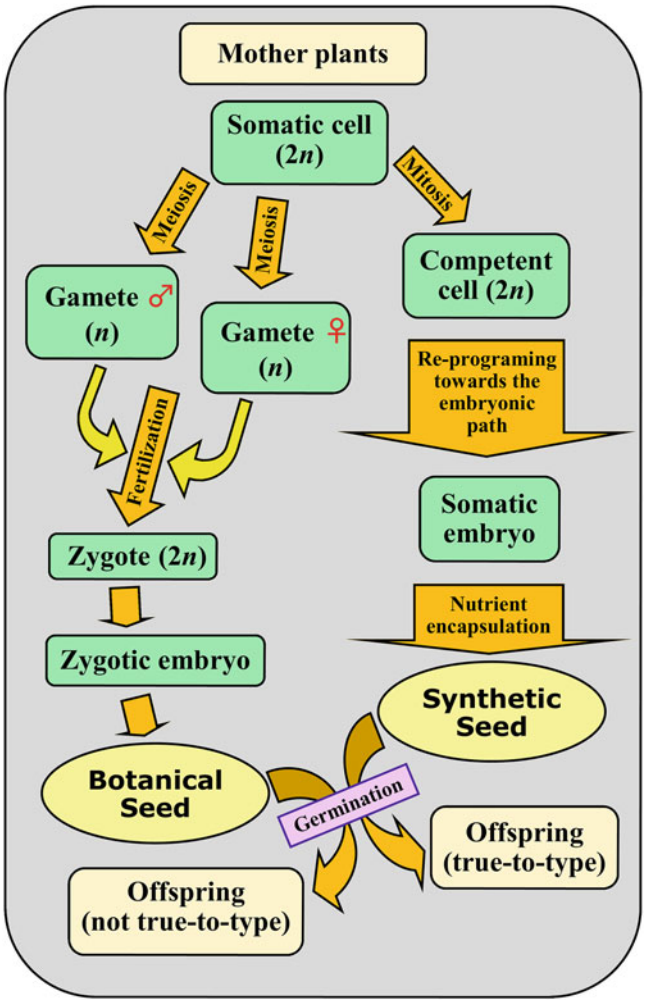

Fig. 9 Schematic representation of the comparative development of zygotic and somatic embryos as well as botanical and synthetic seeds

alternatively known as non-zygotic embryogenesis (Yang and Zhang 2010; El-Esawi 2016). The development of both zygotic and somatic embryos is schematically presented in Fig. 9.

De novo shoot organogenesis is also another good example of in vitro clonal propagation. Dissimilar to somatic embryogenesis, only unipolar (shoot pole) structures are developed during de novo shoot organogenesis (Yumbla-Orbes et al. 2017). The complete plantlets are produced from these de novo shoots pursued by root organogenesis.

7 Clonal Propagation of Genetically Modified (GM) Crops

Transgenic or genetically modified (GM) crops are those plants that have been improved genetically by means of recombinant DNA technology (Key et al. 2008). The population of human beings is growing faster than expected and predicted to reach almost ten billion people by the year 2050, therefore making food security is the vital social issue for the next three decades (Herrera-Estrella 2000). The food availability for everyone, particularly in developing countries, is possible through the cultivation of GM crops (Herrera-Estrella and Alvarez-Morales 2001). In the last two decades, the agricultural lands planted with GM crops have increased more than 100-fold, which clearly indicates that crop biotechnology is today's fast-growing promising area (Mall et al. 2018). America is the chief manufacturer of GM crops, including cotton, maize, soybean, and canola, representing 80% of the entire production of GM crops worldwide (Mall et al. 2018). Bt cotton, Bt brinjal, and GM papaya are well grown in Asian countries (James 2010). Regeneration of plants from the genetically transformed cells is indispensable to the success of genetic engineering and is only possible using in vitro tissue culture techniques (Darbani et al. 2008). The in vitro plant tissue culture system was recently described as "a battle horse in the genome editing" through a novel CRISPR/Cas9 technology (Loyola-Vargas and Avilez-Montalvo 2018). Ones the GM crops introduced through recombinant DNA technology (RDT), cross pollination and subsequent seed formation fail to retain the GM trait. Hence once again, the clonal micropropagation is only a viable option for large-scale production to address the huge requirements of GM crops. Clonal micropropagation of different transgenic plants species like *Betula platyphylla*, *Tylophora indica*, *Lactuca sativa*, and many others is well studied in the last decade (Pua and Davey 2007; Darbani et al. 2008; Zeng et al. 2010; Roychowdhury et al. 2013; Pniewski et al. 2017; Mall et al. 2018).

8 Synthetic Seed: A Modern Approach for Clonal Seed Propagation

Synthetic seeds are nothing but a functional mimic of botanical seeds, which was manufactured in laboratories and therefore alternatively known as manufactured seed or artificial seed (Sharma et al. 2013). Synthetic seed is one of the most promising modern plant biotechnological tools which could be useful for agricultural improvement at present as well as upcoming days. Application of synthetic seed technology is the perfect approach for micropropagation and conservation of important plant species, owing to their several advantages, including genetically true to type nature, comfort in handling and transportation, round-the-year production, and effectiveness in relation to space, time, labour, and cost (Niazian 2019). Recent advancement of in vitro clonal propagation systems opens a door for use of high-

quality high-vigour clonal plants in agri-horticultural field (Anis and Ahmad 2016). However, for commercial application of micropropagation, several steps like large-scale multiplication, in vitro root induction, their acclimatization, and planting are needed, which is more laborious and expensive. In this context, synthetic seed can provide a better option for cost-effective delivery system of in vitro-propagated clonal plants (Sharma et al. 2013) and may prove to be an effective alternative of the botanical seeds in future. Synthetic seeds offer a little-cost, high-volume clonal propagation system (Roy 2013). The advantages of synthetic seeds over other tissue culture-based propagation methods are easy to handle and potential for long-term storages (Rai et al. 2009).

8.1 Concept of Synthetic Seed

The idea of artificial seed or synthetic seed was the brainchild of Japanese botanist Toshio Murashige; he coined the word "artificial seed" for the very first time in 1977 (Murashige 1977). The definition of an artificial seed was first time given by Murashige (1978), as "an encapsulated single somatic embryo, i.e., a clonal product that could be handled and used as a real seed for transport, storage and sowing, and that, therefore, would eventually grow, either in vivo or ex vitro, into a plantlet". Therefore, synthetic seed production was previously restricted to only those plants in which somatic embryogenesis had been successfully standardized. Later, Bapat et al. (1987) proposed to expand the synthetic seed technology to the encapsulation of various in vitro-derived propagules other than somatic embryos, and they used axillary buds of *Morus indica* as a first example of this new application. Up-to-date perusal revealed that more than 20 scientific review papers were already published on the topic "synthetic seed", which clearly reflects the exact importance of this technology in modern days (Table 1). On the basis of research and review papers existent so far, synthetic seeds can be differentiated into two types—(1) encapsulated desiccated synthetic seed and (2) encapsulated hydrated synthetic seed. In botanical seed, after maturity the zygotic embryo enters in dormancy period when all cells of the embryo enter into quiescent (i.e. G_0 phase of the cell cycle) resting phase (Bewley 1997). For the first type, encapsulated desiccated synthetic seed preparation needs desiccation of propagules, which helps to improve the storage capability (or dormancy period) of the synthetic seed by aiding to enter the propagules in quiescent resting phase. However, this type of synthetic seed is less popular because of their low rate of germination as compared to encapsulated hydrated synthetic seed. For the second type, encapsulated hydrated synthetic seeds had to be developed by hydrogel encapsulation of propagule. This method was first time used by Redenbaugh et al. (1984) and was patented by them in 1988 (Patent # 4,780,987). In the present day, hydrogel encapsulation method is the most effective and broadly accepted technique of synthetic seed production (Sharma et al. 2013). Aiming for better understandings on how to prepare synthetic seed more successfully, Rihan et al. (2017b) studied the accumulation of dehydrin proteins during the maturation of

Table 1 Important scientific review papers related to the topic "synthetic seed" or "artificial seed"

Year of publish	Title of the review paper	References
2018	Synthetic seed—future prospects in crop improvement	Chandra et al. (2018). https://ijair.org/administrator/components/com_jresearch/files/publications/IJAIR_2688_FINAL.pdf
2018	Manufactured seeds of woody plants	Hartle (2018). https://doi.org/10.1007/978-3-319-89483-6_8
2017	Artificial seeds (principle, aspects, and applications)	Rihan et al. (2017a). https://doi.org/10.3390/agronomy7040071
2017	The usage of cryopreservation and synthetic seeds on preservation for plant genetic resources	İzgü and Mendi (2017). https://juniperpublishers.com/ijcsmb/pdf/IJCSMB.MS.ID.555583.pdf
2017	Synthetic seed technology in vegetables—A review	Khatoon et al. (2017). http://www.envirobiotechjournals.com/article_abstract.php?aid=7536&iid=224&jid=3
2017	Synthetic seed technology	Magray et al. (2017). https://doi.org/10.20546/ijcmas.2017.611.079
2017	Synthetic seed technology and its applications: A review	Tripathi (2017). http://biotech.journalspub.info/?journal=IJPB&page=article&op=view&path%5B%5D=157
2016	Development of synthetic seed technology in plants and its applications: A review	Nongdam (2016). http://www.currentsciencejournal.info/issuespdf/Nongdam.pdf
2015	Synthetic seed production of medicinal plants: a review on influence of explants, encapsulation agent, and matrix	Gantait et al. (2015). https://doi.org/10.1007/s11738-015-1847-2
2015	Artificial seed: A practical innovation	Panwar (2015). http://www.rroij.com/open-access/artificial-seed-a-practical-innovation.pdf
2014	Synthetic seeds: A boon for conservation and exchange of germplasm	Kumara et al. (2014). http://advancejournals.org/bmr-biotechnology/article/synthetic-seeds-a-boon-for-conservation-and-exchange-of-germplasm/
2013	Synseed technology—a complete synthesis	Sharma et al. (2013). https://doi.org/10.1016/j.biotechadv.2012.09.007
2013	Synthetic seed production; its relevance and future panorama	Siddique et al. (2013). https://doi.org/10.21276/ajptr
2012	Production and applications of artificial seeds: A review	Ravi and Anand (2012). http://www.isca.in/IJBS/Archive/v1/i5/13.ISCA-JBS-2012-106.php
2012	Synthetic seeds: A review in agriculture and forestry	Reddy et al. (2012). https://academicjournals.org/journal/AJB/article-full-text-pdf/FEF310B30197
2011	Alginate-encapsulated shoot tips and nodal segments in micropropagation of medicinal plants. A review	Kikowska and Thiem (2011)

(continued)

Table 1 (continued)

Year of publish	Title of the review paper	References
2011	The green revolution via synthetic (artificial) seeds: A review	Helal (2011). http://www.aensiweb.net/AENSIWEB/rjabs/rjabs/2011/464-477.pdf
2009	The encapsulation technology in fruit plants—A review	Rai et al. (2009). https://doi.org/10.1016/j.biotechadv.2009.04.025
2001	Artificial seeds and their applications	Saiprasad (2001). https://www.ias.ac.in/article/fulltext/reso/006/05/0039-0047
2000	Synthetic seeds: a novel concept in seed biotechnology	Bapat (2000). http://www.barc.gov.in/publications/nl/2000/200009-02.pdf
2000	Synthetic seed: prospects and limitations	Ara et al. (2000). https://www.jstor.org/stable/24104316
1998	Recent perspectives on synthetic seed technology using non-embryogenic in vitro-derived explants	Standardi and Piccioni (1998) https://www.jstor.org/stable/10.1086/314087
1993	Embryogeny of gymnosperms: advances in synthetic seed technology of conifers	Attree and Fowke (1993). https://doi.org/10.1007/BF00043936
1992	Artificial seeds	Senaratna (1992). https://doi.org/10.1016/0734-9750(92)90301-O
1991	Somatic embryogenesis and development of synthetic seed technology	Gray et al. (1991). https://doi.org/10.1080/07352689109382306

botanical seeds of cauliflower and their significant role in the drought tolerance of seeds, and these findings could help on quality improvement of artificial seeds.

8.2 *Considerable Criteria in the Designing of Synthetic Seed*

At the time of synthetic seed preparation, the following three basic properties of botanical seeds have to be fulfilled—(1) primarily it must contain a propagule which later grows up as a plantlet (like zygotic embryo in botanical seed), (2) it should contain a nutrient medium which serves as storage food for plant propagule (like endosperm or cotyledons in botanical seed), and (3) the plant propagule should be covered by a hard protective layer for mechanical protection (like seed coat in botanical seed) (Fig. 10).

8.3 *Preparation of Synthetic Seed*

Somatic embryo is the ideal propagule for encapsulation to produce synthetic seed because of its bipolar nature (Gray et al. 1991; Ghosh and Sen 1991, 1994), but

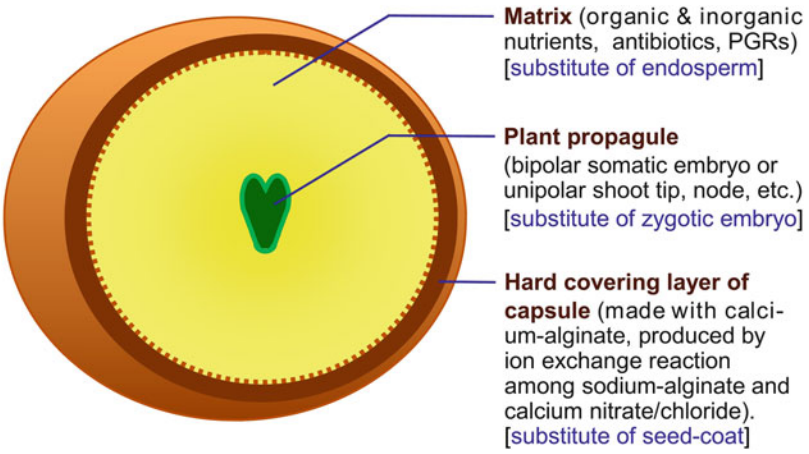

Fig. 10 Diagrammatic representation of the concept of synthetic seed and their different parts

successful somatic embryogenesis protocol is not established in many important plant species. Alternatively, any parts of plant which have the ability to grow can be used as non-embryogenic propagule (Fig. 11). In this context, recently different parts of plant organs like shoot tip and node (containing apical and axillary shoot buds), protocorm-like bodies (PLBs), corm, rhizome, micro-bulblet, micro-tuber, etc. are very popularly used for synthetic seed preparation (Fig. 12 and Table 2).

Nutrients present in encapsulation matrix are used by encapsulated plant propagule for their nutritional requirement. Different formulations of basic nutrient media which are used for in vitro plant culture are also used in encapsulation matrix with slight modification (Gantait et al. 2015). Although all the basic nutrients are same, calcium salts are not added. For example, if MS nutrients (Murashige and Skoog 1962) are used in encapsulation matrix, then calcium chloride is replaced with sodium chloride, which is devoid of calcium ions but fulfils the requirement of chloride ions. Apart from inorganic nutrients and sodium alginate, carbohydrates in the form of sucrose or glucose are also needed. In addition to inorganic and organic nutrients, plant growth regulators, antibiotics are also used in encapsulation medium (Sharma et al. 2013).

Another important requirement of encapsulation matrix is hydrogel. More than a few encapsulating agents such as agarose, potassium alginate, sodium alginate, gelrite, sodium pectate, sodium alginate with carboxymethyl cellulose, guar gum, carrageenan, tragacanth gum, gelatin, etc. have been experimented as hydrogels (Ara et al. 2000; Rai et al. 2009; Sharma et al. 2013). Among all of these gelling agents, sodium alginate achieved maximum popularity due to its adequate viscosity, rapid gelation, low cost, as well as non-toxicity for plants (Gantait et al. 2015).

For the preparation of encapsulated hydrated synthetic seeds, the encapsulation medium (or matrix of synthetic seed) should be prepared at first. Encapsulation medium contains all inorganic and organic nutrients (without calcium ion) of any

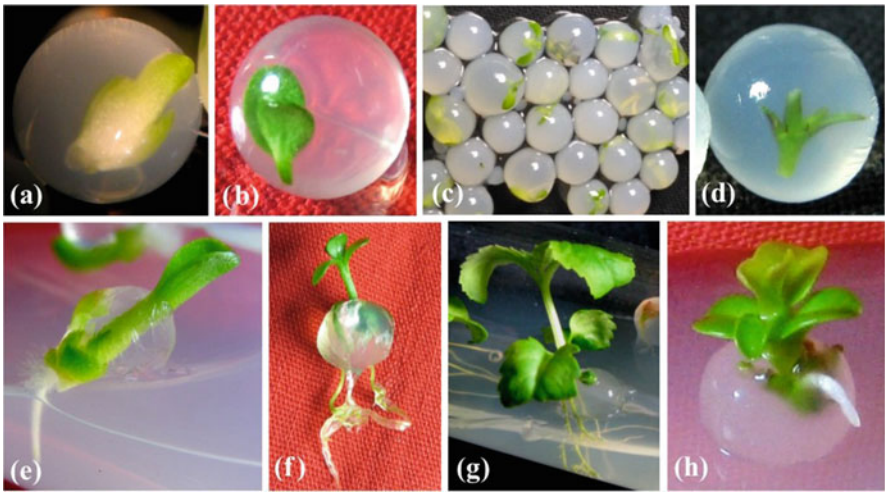

Fig. 11 Synthetic seeds prepared from embryonic and non-embryonic explants. (**a**–**d**) Freshly prepared seeds produced by encapsulating the somatic embryo, shoot-tips, and nodal segment, respectively. (**e**–**h**) germinated seeds, (**a**, **e**) synthetic seed of *Ledebouria revoluta* before and after germination, (**b**, **f**) synthetic seed of *Bacopa monnieri* before and after germination, (**c**, **g**) synthetic seed of *Bacopa chamaedryoides* before and after germination, and (**d**, **h**) synthetic seed of *Tylophora indica* before and after germination

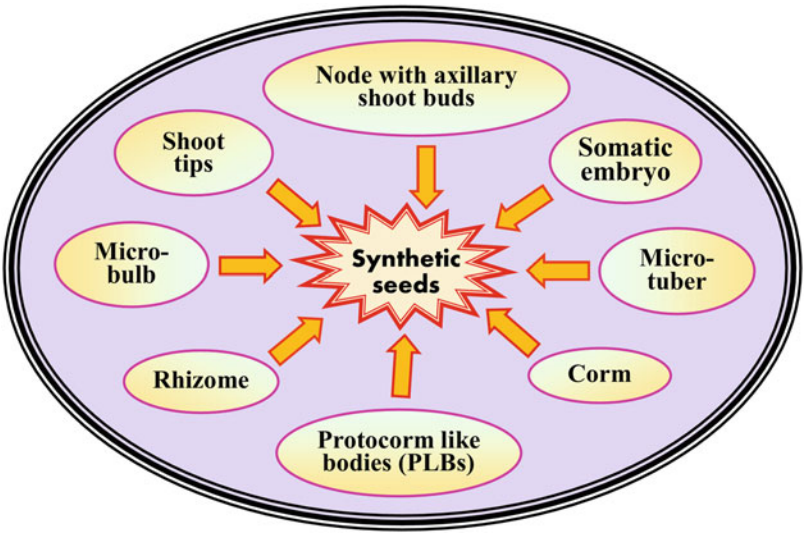

Fig. 12 Schematic representation of different types of explant used for synthetic seed preparation

Table 2 Important publications (in 2016 and onwards) on synthetic seed informing about the recent trends on use of different explants for encapsulation

Year of publish	Plant species	Encapsulated explant type	References
2016	*Solanum tuberosum*	Axillary buds	Ghanbarali et al. (2016). https://doi.org/10.1007/s11240-016-1013-6
2018	Sugarcane	Bud chip	da Silva et al. (2018). https://doi.org/10.5539/jas.v10n4p104
2018	Sugarcane	Micro-shoots	Badr-Elden (2018). https://ejbo.journals.ekb.eg/article_5168_2f3913c2d70d6a019aa587b3a90fd465.pdf
2018	*Althaea officinalis*	Nodal segments	Naz et al. (2018). https://doi.org/10.1080/11263504.2018.1436610
2018	*Capparis decidua*	Nodal segments	Siddique and Bukhari (2018). https://doi.org/10.1007/s10457-017-0120-7
2018	*Ceropegia barnesii*	Nodal segments	Ananthan et al. (2018). https://doi.org/10.1007/s11627-018-9934-x
2017	*Erythrina variegata*	Nodal segments	Javed et al. (2017). https://doi.org/10.1016/j.indcrop.2017.04.053
2016	*Manihot esculenta*	Nodal segments	Hegde et al. (2016). http://isrc.in/ojs/index.php/jrc/article/view/407/290
2018	*Salix tetrasperma*	Nodal segments	Khan et al. (2018). https://doi.org/10.1016/j.bcab.2018.07.002
2018	*Sphagneticola calendulacea*	Nodal segments	Kundu et al. (2018). https://doi.org/10.1007/s11738-018-2633-8
2017	*Tylophora indica*	Nodal segments	Gantait et al. (2017b). https://doi.org/10.1016/j.hpj.2017.06.004
2017	*Vitex trifolia*	Nodal segments	Alatar et al. (2017). https://doi.org/10.1080/14620316.2016.1234949
2017	*Spathoglottis plicata*	PLBs	Haque and Ghosh (2017b). https://doi.org/10.1016/j.hpj.2017.10.002
2018	*Ansellia africana* (Leopard orchid)	Protocorm-like bodies (PLBs)	Bhattacharyya et al. (2018). https://doi.org/10.1007/s11240-018-1382-0
2018	*Plumbago rosea*	Shoot tips	Prakash et al. (2018). https://doi.org/10.1007/s12298-018-0559-7
2017	*Rauvolfia serpentina*	Shoot tips	Gantait et al. (2017a). https://doi.org/10.1016/j.jarmap.2017.01.005
2017	*Rauvolfia serpentina*	Shoot tips	Gantait and Kundu (2017). https://doi.org/10.1007/s12210-017-0637-8
2018	*Taraxacum pieninicum*	Shoot tips	Kamińska et al. (2018). https://doi.org/10.1007/s11240-017-1343-z
2018	*Urginea altissima*	Shoot tips	Baskaran et al. (2018). https://doi.org/10.1007/s13205-017-1028-7
2018	*Nerium oleander*	Shoot tips and first nodal segments	Hatzilazarou et al. (2018). https://doi.org/10.1080/14620316.2018.1542283
2017	*Withania coagulans*	Shoot tips and Nodal segments	Rathore and Kheni (2017). https://doi.org/10.1007/s40011-015-0577-y

(continued)

Table 2 (continued)

Year of publish	Plant species	Encapsulated explant type	References
2016	*Zingiber officinale*	Shoot tips and Somatic embryos	Babu et al. (2016). https://doi.org/10.1007/978-1-4939-3332-7_28
2016	Mountain garlic	Shoot tips or Micro-bulbs	Mahajan (2016). https://doi.org/10.1007/978-1-4939-3332-7_23
2016	*Citrus* spp.	Somatic embryo	Micheli and Standardi (2016). https://doi.org/10.1007/978-1-4939-3061-6_30
2016	*Bacopa monnieri*	Somatic embryos	Khilwani et al. (2016). https://doi.org/10.1007/s11240-016-1067-5
2016	*Curcuma amada*	Somatic embryos	Raju et al. (2016). https://doi.org/10.1007/s13580-016-0096-7
2017	Date palm	Somatic embryos	Bekheet (2017). https://doi.org/10.1007/978-1-4939-7159-6_7
2016	*Ledebouria revoluta*	Somatic embryos	Haque and Ghosh (2016b). https://doi.org/10.1007/s11240-016-1030-5

suitable formulation of basic medium (e.g. MS medium), sucrose, sodium alginate, plant growth regulators, and antibiotics. Suitable plant propagules are mixed with encapsulation medium, and then sodium alginate-containing medium dropped into a solution of calcium salt (calcium chloride or calcium nitrate). Each drop (bead) containing a single propagule should be maintained in calcium solution for 10–20 min with gentle shaking. Ion exchange reaction takes place on the outer surface of the beads where Na^{+} ion of the sodium alginate is replaced with Ca^{+2} to form a hard layer of calcium alginate (Jha and Ghosh 2016). Polymerization of calcium alginate resulted in the construction of hydrogel capsules with single propagule inside—i.e. synthetic seed. The capsules containing gel matrix actually perform as a repository of nutrient which assists in the survival, as well as growth and development of propagules (Gantait et al. 2015).

9 Applications of Synthetic Seed to Avoiding Heterozygosity

Applications of synthetic seed are not only restricted to avoiding the heterozygosity problem but have a wide-ranging list of utility (Fig. 13). In general, synthetic seed technology is utilized for the following purposes—(1) for micropropagation; (2) for short-, medium-, or long-term conservation; (3) for clonal or true to type seed production; (4) for large-scale seed production aimed at commercial use; (5) for season-independent round-the-year seed production; (6) for propagation of non-seed-bearing plants; (7) for easy handling and transportation; and (8) for exchange of germplasm between different countries by lowering plant quarantine requirements as for the germ-free condition of the plant propagules (Ara et al. 2000; Rai et al. 2009; Sharma et al. 2013; Hartle 2018; Chandra et al. 2018).

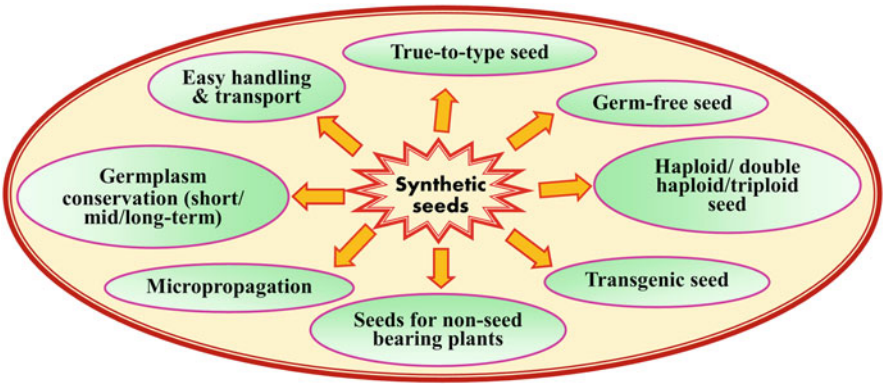

Fig. 13 Schematic representation of different applications of synthetic seeds

Now keeping aside the general benefits, let us come back to our main focus, i.e. avoiding heterozygosity through synthetic seeds. Two phenomena, namely, meiotic crossing over and gametic fusion, are unavoidable events of sexual reproduction. In diploid plants, one chromosome of each homologous pair originates from male parent and another from female parent. During crossing over, the genetic materials of two different chromosomes of the homologous pairs get mixed and recombined due to the reciprocal exchange among non-sister chromatids (Schwarzacher 2003). As a result, new types of heterozygosity are generated in haploid gametic cells. Now another event is gametic fusion where a lot of new forms of heterozygosity are generated. During fertilization, male and female gametes may perhaps from two different parents participate; as a result two totally different sets of alleles come together in zygotic cell (Acquaah 2012). Hence, in comparison with any one of the parental plants, the newly formed zygote contains many new combinations of alleles, i.e. so many new forms of heterozygosity are created in zygote.

Since all the propagules used for synthetic seed preparation are propagated vegetatively, which means they escape meiotic recombination (during crossing over) as well as gametic fusion of two different parental genomes (Clift and Schuh 2013), they ceased the chances of the formation of new types of heterozygosity in seed. Although the heterozygous conditions which are already present in source plant (from where clonal propagations initiated) can't be eliminated, they can be shifted to all offspring.

10 Summary and Future Prospects

Synthetic seed is an up-to-date tool of plant biotechnology which manufactured in laboratories and serve as efficient alternative of botanical seeds. Since vegetatively propagated propagules are used for synthetic seed preparation, all the manufactured

seeds are genetically true to type of their parent. Nevertheless, the heterozygosity that already existed in a mother plant cannot be eliminated but can also be transmitted in all synthetic seed-derived offspring.

However, if the production of homozygous synthetic seeds is wanted in realistic form, the only method is double haploid source plant selection, because double haploid plants are always truly homozygous (Prigge et al. 2012; Kleter et al. 2019). The clonal propagation of double haploid plant gives rise to a huge number of homozygous clones. If those clonal propagules are used for synthetic seed preparation, then the heterozygosity problem will totally be avoidable. Otherwise synthetic seed technology can only help to restrict the construction of new forms of heterozygosity in successive regeneration cycle, which are abundant in zygotic or botanical seeds.

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Insight View of Topical Trends on Synthetic Seeds of Rare and Endangered Plant Species and Its Future Prospects

B. Nandini and P. Giridhar

Abstract In the present era, global plant biodiversity is dilapidated annually at a pioneering rate. Preservation of plant genetic resources has been threatened by the safety with foremost problems by limiting its efficiency. Contemporary trends on synthetic seeds, i.e., artificially encapsulated somatic embryos, open up new avenue in agriculture. It is one of the most promising alternate tools for propagation of many rare and endangered plant species. Success of synthetic seeds endowed with its protective hydrogel coating by increasing the growth of micropropagule in the field conditions. Synthetic seeds are also helpful in stipulations of their role in preventing the spread of plant diseases, and it is a gifted path for scale-up of multi-clone production for commercial purpose. Synthetic seeds are more durable for handling, transportation, and storage. Presently, it is well documented that any kind of non-embryogenic explants from tissue culture can be used for synthetic seed production like shoot tips, axillary buds, nodal segments, protocorms, bulblets, section of callus, bipolar propagule, or unipolar propagule. Currently, significant focus has been given for synthetic seeds, as they are economical for conservation of germplasm, maintenance of the genetic uniformity of plants through clonal propagation, and direct deliverance to the field. Under this context, various effective protocols for preparing synthetic seeds and conservation of plants are developed. Accordingly, an appraisal on synthetic seed aspects of various endangered and rare plant species has been contemplated, and the same will be covered in this chapter.

Keywords Synthetic seeds · Explants · Tissue culture · In vitro propagation · Encapsulation

Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
BAP	6-Benzylaminopurine

B. Nandini · P. Giridhar (✉)
Plant Cell Biotechnology Department, CSIR-CFTRI, Mysuru, Karnataka, India

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$Ca(NO_3)_2$	Calcium nitrate
$CaCl_2$	Calcium chloride
IAA	Indole-3-acetic acid
MS	Murashige and Skoog medium
NAA	α-Naphthalene acetic acid
PLBs	Protocorm-like bodies
TDZ	Thidiazuron
TTC	Triphenyltetrazolium chloride

1 Introduction

Plant genetic diversity is threatened globally due to unparalleled perturbations, habitat loss, and extinction pace. Various species are depicted as rare and endangered due to unprogrammed nature of work. Presently, integrated programs are necessary to protect and preserve biodiversity in its natural habitat (Sarasan et al. 2006). Synthetic seed technology is an exhilarating and swiftly budding area of research as it compacts with conservation and storage of enviable genotypes besides its easy handling and transportation (Mohanty et al. 2013). Synthetic seeds are generally referred as "syn" seeds and described as artificially encapsulated somatic embryos, shoot buds, cell aggregates, or any other tissue that can be exercised for sowing as a seed and that owns the ability to transfer into a plant underneath in vitro or ex vitro conditions and further retains its potential after storage (Capuano et al. 1998). In the present era, the approach of synthetic seeds for ex situ preservation of the germplasm of elite endangered plant species was strongly emphasized (Rao 2004; Borner 2006). An invention of synthetic seeds facilitates in minimizing the price of micropropagated plantlets economically. In most of the trees, seed propagation has not been successful because of reduced endosperm, an incidence of heterozygosity of seeds, and low germination rate. This circumstance raises the interest on encapsulation technology for propagation and conservation of germplasm.

Synthetic seed technology is stated as a boon for conservation and exchange of germplasm (Kumari et al. 2014). Synthetic seeds are novel analogue to true seed comprising of a somatic embryo enclosed by an artificial coat which is almost similar to an immature zygotic embryo, probably at early cotyledonary stage (Bekheet 2006). This artificial seed technique was primarily applied in clonal propagation to nurture somatic embryos set into an artificial endosperm and restrained by an artificial seed coat. Potential advantages of synthetic seeds involve their term as "genetically identical materials" with increased competence of in vitro propagation in terms of space, time, labor, and overall cost (Nyende et al. 2003). Owing to these advantages, there is an increased attention in using encapsulation technology in various plant species. Synthetic seeds possess immense potential for large-scale production of plants at low cost value as an alternative option to true seeds (Roy and Mandal 2008). In vitro techniques are known as key factors of biotechnological

advances boasting potential to regenerate elite genotypes and to preserve important plant genetic resources (Rathore and Kheni 2015). The modern progress in encapsulation technology provide diverse potential attributes, such as production of synthetic seed as a potential substitute for mass production of plantlets for different practices and through encapsulation approach the exchange of germplasm among different laboratories in the form of encapsulated alginate micro-cuttings (Micheli et al. 2007; Sharma et al. 2013). Potential of plantlet conservation by retention and regeneration is most enviable property of encapsulated beads after storage (Micheli et al. 2007; Parveen and Shahzad 2014). Momentous success was achieved in the past few years by encapsulation of both embryogenic and non-embryogenic in vitro derived plant material (Ara et al. 2000; Lambardi et al. 2006; Gantait et al. 2012; Gantait and Sinniah 2013; Sharma et al. 2013). Apparently, 3% sodium alginate solution and 100 mM $CaCl_2$ facilitated as most suitable ion exchange involving Na^+ and Ca^{2+}, producing transparent, compact, isodiametric beads (Gantait et al. 2015). To overcome the extinction of valuable plant species in nature, more emphasis has to be given to improve the protocol of synthetic seed in view of long-term conservation of elite germplasm to reintroduce in its original habitat.

2 Endangered Plant Species Used for Synthetic Seed Production (Table 1)

2.1 Centaurium rigualli Esteve.

Centaurium rigualli Esteve. commonly known as "stomach bitter" which belongs to the family Gentianaceae is endemic to the Southeast of the Iberian Peninsula and recorded as an "endangered" according to the IUCN grouping, owing to its minute population range and threats to its habitat from urban development (Alcaraz et al. 1987). It is a distinctive case of a species with low seed accessibility due to its small population. The conservation of this species by in situ was found to be not so easy, because of the human pressure in the region of its natural habitat (Alcaraz et al. 1987). Conservation approaches in terms of tissue culture and micropropagation by synthetic seed path are not yet channelized properly. However, nodal segments from in vitro grown shoots of the *C. rigualii* were effectively cryopreserved. A protocol was developed for improving survival after direct immersion in liquid nitrogen (−196 °C) by the encapsulation-dehydration method to safeguard against ice crystal development. Gonzalez-Benito et al. (1997) able to achieve 70% survival of *C. rigualli* synthetic beads after 8 weeks in culture on semisolid regeneration medium, i.e., liquid MS medium comprising with 1 mg L^{-1} BAP plus 0.1 mg L^{-1} NAA. In this case, synthetic seeds were prepared with alginate beads 3% in MS liquid medium having medium viscosity, amid with sucrose, 0.75 M and 100 mM $CaCl_2$. Other than above reports, there are no accessible studies on the regeneration of *C. rigualli* by in vitro propagation as well as its conservation strategies. Hence, there is an imperative need to focus on this plant propagation and long-term conservation approaches to avoid extinction.

Table 1 List of endangered plant species used for synthetic seed production

Plant species	Importance	Family	Synthetic medium	Explant source	References
Centaurium rigualli Esteve.	Endangered plant species	Gentianaceae	3% sodium alignate + 100 mM $CaCl_2$	Nodal segments	Gonzalez-Benito et al. (1997)
Plantago algarbiensis Samp.	Endangered endemic species from the Algarve	Plantaginaceae	3% sodium alginate + 100 mM $CaCl_2$ solution	Nodal segments	Coelho et al. (2014a)
Rhododendron maddeni Hook. f.	Endangered plant	Ericaceae	3% sodium alginate + 60 mM $CaCl_2{\cdot}2H_2O$	Shoot tips	Singh (2008)
Splachnum ampullaceum Hedw.	Endangered moss	Splachnaceae	1% low-viscosity sodium alginate + 100 mM $CaCl_2$ solution	Moss buds	Mallon et al. (2007)
Sterculia urens Roxb.	Endangered Gum-yielding tree	Sterculiaceae	4% Sodium alginate + 0.5 mg L^{-1} 6-benzyl adenine + 1.0 mg L^{-1} Indole-3-butyric acid + 100 mM $CaCl_2{\cdot}2H_2O$	Nodal segments	Devi et al. (2014)
Taraxacum pieninicum Pawł.	Endangered plant species	Asteraceae	3% sodium alginate + 100 mM $CaCl_2{\cdot}2H_2O$	Microshoots/ shoot tips	Kamińska et al. (2018a) Kamińska et al. (2018b)

2.2 *Plantago algarbiensis Samp.*

Plantago algarbiensis belongs to Plantaginaceae family, is an endemic and endangered species to the Algarve, Portugal, due to populaces restricted to a quite small area. Various species of *Plantago* are scheduled as harmless herbs in the pharmacopeias of several countries (Blumenthal 1998), whereas few are utilized as animal feed and food purposes (Chiang et al. 2003). *P. algarbiensis* is a rosulate hemicryptophyte (Franco 1984). Due to its constrained allocation worldwide, *P. algarbiensis* is in threat of extinction globally and is considered endangered species with legal protection by the Portuguese law and European Habitats Directive 92/43/CEE (reference 140/99 of April 24; ICN 2007). Micropropagation by in vitro practice has been reported, as conservation approach of ex situ for safeguarding *P. algarbiensis*. Shoot buds of *P. algarbiensis* was propagated successfully and acclimatized effectively under ex vitro condition with 95% of survival rate (Goncalves et al. 2009). Synthetic seed approach for the conservation of *P. algarbiensis* was performed by cryopreservation of nodal segments in droplet-vitrification and encapsulation-dehydration method. To obtain encapsulated nodal segments, sodium alginate 3% prepared in MS medium with 0.35 M sucrose and 100 mM $CaCl_2$ solution were used. Encapsulation-dehydration method reveals the significant percentage of regrowth, i.e., 63% after 3-h desiccation on MS medium amended with 0.2 mg L^{-1} BA (Coelho et al. 2014a). Further there are no inclusive studies on in vitro propagation and synthetic seeds of *P. algarbiensis.* Rapid clonal propagation and synthetic seed formulation via encapsulation have to be performed for its long-term conservation and reintroduction to the natural haunt.

2.3 *Rhododendron maddeni Hook. f.*

The genus Rhododendron is a member of the family Ericaceae. They are the residents of elevated altitude, including about 1000 species typically dwelling in a cosmic part of Southeastern Asia extending from Northwestern Himalaya all the way through Nepal, Northeastern India, Eastern Tibet, Northern Burma, and Western and Central China (Leach 1961; Pradhan and Lachungpa 1990). *Rhododendron maddeni* Hook. f. is an endangered and beautiful rhododendron that has restricted allocation. During the past few years, modest attempts for in vitro restoration have been made for this endangered species by using nodal and shoot tip segments (Mercure et al. 1998; Kumar et al. 2004; Singh and Gurung 2009; Eeckhaut et al. 2010). The synthetic seeds or artificial seed production by encapsulation technique has become a significant asset in micropropagation of this plant. Synthetic seeds of *R. maddeni* were developed under in vitro condition by encapsulation of shoot tips with 3% sodium alginate in Anderson medium with 3% sucrose and 60 mM $CaCl_2 \cdot 2H_2O$. Further, these synthetic seeds can be preserved for 30 days at a temperature of 5 °C, and later 68% of shoot proliferation noticed on Anderson medium supplemented with

7 mg L^{-1} isopentenyladenine, 100 mg L^{-1} polyvinyl pyrrolidone, 100 mg L^{-1} ascorbic acid, 10 mg L^{-1} citric acid, 3% sucrose, and 3% phytagel was recorded. Upon transplantation to ex vitro conditions, 86% survival frequency of plantlets was recorded (Singh 2008). To date, no further significant effort has been made on the advance of synthetic seeds for mass scale production of rhododendron species, which is beneath the threat of extinction.

2.4 *Splachnum ampullaceum Hedw.*

The *Splachnum ampullaceum* Hedw. commonly known as dung moss and belongs to the family Splachnaceae is a critically endangered moss in the Iberian Peninsula (Sergio et al. 1994; Red Data Book of European Bryophytes, ECCB 1995; Reinoso et al. 2002). In bryophytes, in vitro culture methods are assured to be helpful for medium and long-period germplasm conservation (Gonzalez et al. 2006). In vitro regeneration and long-term conservation approach of *S. ampullaceum* germplasm have been illustrated by using protonema explants. Enduring conservation of *S. ampullaceum* has been examined via deliberate growth by modifying the compounds in culture medium (Gonzalez et al. 2006). Mallon et al. (2007) studied encapsulation parameter of *S. ampullaceum* with calcium alginate matrix consisting of 1% (w/v) low-viscosity sodium alginate solution dropped into $CaCl_2$, and further they evaluated the efficiency of the moss regeneration with an aptitude of short-, medium-, and long-term periods of conservation by preserving the species via cryopreservation. They suggested encapsulation of moss with no prior treatment is suitable approach for long-period germplasm conservation with as high as 50% survival rates achieved in cold storage even after 2.5 years. Long-term preservation of *S. ampullaceum* was poised with the preservation of the beads in a minute amount of water in containers during slight dehydration, when the inner atmosphere in the alginate beads was a hypertonic solution. To conserve this endangered moss, still more standard protocol for in vitro propagation and synthetic seeds techniques has to be developed.

2.5 *Sterculia urens Roxb.*

Sterculia urens is an endangered gum-yielding tree, which belongs to the family Sterculiaceae, and it is an average-sized tree that grows in the deciduous forests of India. It is a native inhabitant of tropical Himalayas and distributed widely in different states of India, viz., Assam, Bihar, Eastern and Western Peninsula, northeast of Belgaum, Maharashtra and Southern Gujarat (Kumar 2016; Kumar and Desai 2016), Sri Lanka, Australia, Pakistan, Panama, and Malaysia. The gum of *S. urens* is in huge demand both inside and outside India (Anonymous 1976) as a wet-end additive in the manufacture of paper in concurrence with starches. Naturally, the

S. urens propagation is through seeds, but the seed viability declines as the timescale progresses (Devi et al. 2012). Since the tree turns into recalcitrant, its propagation via stem cuttings from mature plants creates difficulties (Thorpe et al. 1991). Further, as it grows the rooting percentage and organogenic potential decline (Mascarenhas and Muralidhan 1989). Micrografting methods will be used in such cases (Amiri 2005). However, such method doesn't give successful results in *S. urens* (Sunnichan et al. 1998). The tree has become endangered owing to overexploitation. Micropropagation of *Sterculia urens* has been carried out for the large-scale propagation from nodal segments (Purohit and Dave 1996; Sunnichan et al. 1998) and intact seedlings (Hussain et al. 2008). Synthetic seeds were developed for conserving the *S. urens* germplasm by encapsulating the explants of nodal segment in alginate droplets consisting of 4% sodium alginate with 100 mM $CaCl_2 \cdot 2H_2O$ and illustrated efficiency of developed encapsulated synthetic buds for in vitro propagation as well as ex situ conservation. Encapsulated nodal segments show significant regeneration potential (73.33% ± 1.33) on MS + TDZ (0.2 mg L^{-1}) even after storage of 6 months at 4 °C (Devi et al. 2014). The dominance of TDZ was reported in encapsulated axillary buds of *S. urens* (Devi et al. 2011). Advanced research is required in view of *S. urens* to ideal the technology so that it can be utilized on a commercial range.

2.6 *Taraxacum pieninicum Pawł.*

Taraxacum pieninicum Pawł. is the oldest critically endangered endemic plant arises in the Pieniny Mountains (Poland). It belongs to the prime family of flowering plants, Asteraceae. Features like a minute amount of entity with inadequate germination of seeds due to the feeding of invertebrates, particularly snails, are threat factor to the endurance of this plant species. Additionally, *Taraxacum* species seeds are hard to preserve, as seed germination takes place directly after maturation and their significant loss of seed vigor after storage of 2 years (Tas and Van Dijk 1999; Honek et al. 2005). A procedure for competent in vitro micropropagation and process for preserving the shoot tips of *T. pieninicum* species at 10 °C were demonstrated (Trejgell et al. 2013; Kamińska et al. 2016). But, growth of the shoot has not been suitably repressed despite the low storage temperature, which negatively influenced the value of the stored material quality. Kamińska et al. (2018a) overviewed on inhibition *T. pieninicum* shoot growth by low temperature at 4 °C and an advance approach to limit the harm on meristem tissue through synthetic seed formation via alginate encapsulation of the shoot tips. Microshoots are encapsulated with 3% sodium alginate primed in MS liquid medium and 100 mM $CaCl_2 \cdot 2H_2O$. Synthetic seed confirms conversion frequency of 70.0–96.7% regardless of the duration period on full-strength MS medium with 3% sucrose plus 0.14 μM NAA, 1.11 μM BA, and 0.8% agar in preeminent growth parameters. They illustrated that *T. pieninicum* synthetic seeds prepared from shoot tips can be preserved even for 12 months without subculture at 4 °C. Synthetic seed storage is most suitable in dark conditions with significant survival, visual, and proliferation

rate. Further, influence of jasmonic acid on cold storage of encapsulated shoot tips of *T. pieninicum* with 3% sodium alginate primed in liquid MS medium and 100 mM $CaCl_2 \cdot 2H_2O$ was examined by Kamińska et al. (2018b). Application of jasmonic acid exogenously slightly induces cold tolerance, and further tissue growth inhibition is more effective compared to subsequent preculture on media amended with jasmonic acid (Kamińska et al. 2018b). In view of these points, it conveys that still even more appropriate procedure for long-term preservation of *T. pieninicum* has to be developed with standard program to evade the extinction of this species.

3 Medicinally Important Endangered Plant Species Used for Synthetic Seed Production (Table 2)

3.1 Arnebia euchroma (Royle) Jonst.

Arnebia euchroma (Royle) Jonst. belongs to the family of Boraginaceae and is a perennial herb of the Alpine province. It is found in the regions of Pamirs, north and south hillside of the Tian Shan Mountain in Xinjiang, Western Tibet, Pakistan, Nepal, Iran, Afghanistan, the Central Asia area of the Soviet Union, and Siberia the Himalaya above the sea level between a range of altitude 3700 and 4200 m (Anonymous 1985). Because of its remedial importance, the species is being collected subjectively from the native habitat for pharmaceutical and domestic purposes. This effected in status of *A. euchroma* as critically endangered and has recorded in the species list for protection and conservation in West Himalaya (Molur and Walker 1998). Since ancient periodic times, derivatives extorted from the *A. euchroma* roots exercised as dyes for silk, used in traditional medicine and food products and further shikonin valued at US$4000 per kg (wholesale price) (Kaith et al. 1996; Kosger et al. 2009). Tissue culture of *A. euchroma* was initially payed attention in Russia (Davydenkov et al. 1991). Further, protocols have been developed for in vitro shoot multiplication of *A. euchroma* using thidiazuron (Jiang et al. 2005; Malik et al. 2016). Most of the tissue culture studies of *A. euchroma* have been payed attention much on enhancing shikonin and other metabolite production (Zakhlenjuk and Kunakh 1998; Gupta et al. 2014; Arghavani et al. 2015). Simultaneous organogenesis, somatic embryogenesis, and synthetic seed production have been exemplified in *A. euchroma* by encapsulating early cotyledonary-stage embryos with sodium alginate (3%) and $Ca(NO_3)_2$ (for 25 min, 100 mM) under in vitro condition, and it shows 60.6% germination rate in MS medium. Further, rooted shoots showed 72% survival rate under ex vitro conditions (Manjkhola et al. 2005). Still even much more work has to be done on synthetic seed concept of *A. euchroma* for conservation and long-term preservation of germplasm. Appliance of efficient synthetic seed protocols is extremely helpful to maintain the resource in its natural inhabitants and also to preserve for long-term storage of elite genotypes.

Table 2 List of medicinally important endangered plant species used for synthetic seed production

Plant species	Importance	Family	Synthetic medium	Explant source	References
Arnebia euchroma (Royle) Jonst.	Endangered medicinal plant	Boraginaceae	3% sodium alginate + 100 mM $Ca(NO_3)_2$	Early cotyledonary-stage embryos	Manjkhola et al. (2005)
Ceropegia spiralis Wt. and *Ceropegia pusilla* Wt. and Arn.	Endangered and endemic medicinal herbs	Asclepiadaceae	3% sodium alginate + 50 mM $CaCl_2$ solution	Shoot tips, nodal segments	Murthy et al. (2013)
Dendrobium nobile Lindl.	Endangered medicinal orchid with commercial and ornamental importance	Orchidaceae	3% sodium alginate + 7.5% mannitol + 7.5% sucrose + 100 mM $CaCl_2{\cdot}2H_2O$ solution	PLBs	Mohanty et al. (2013)
Rotula aquatica Lour.	Rare rhoeophytic woody medicinal plant	Boraginaceae	3% sodium alginate + 50 mM $CaCl_2$ solution	Cotyledonary-stage somatic embryos	Chithra et al. (2005)
Spilanthes mauritiana DC.	Endangered medicinal herb	Asteraceae	3% and 4% sodium alginate + 100 mm $CaCl_2{\cdot}2H_2O$	Nodal segments	Sharma et al. (2009a) Sharma and Shahzad (2014)
Swertia chirayita (Roxb. ex Fleming. H. Karst.)	Endangered medicinal herb	Gentianaceae	4% sodium alginate + 100 mM $CaCl_2{\cdot}2H_2O$ solution	Torpedo stage somatic embryos	Kumar and Chandra (2014)
Tecomella undulata (Sm.) Seem.	Endangered economically and pharmaceutically important tree	Bignoniaceae	3% sodium alginate + 100 mM $CaCl_2{\cdot}2H_2O$ + 2.5 μM BA + 0.5 μM IAA	Nodal explants	Shaheen and Shahzad (2015)
Tuberaria major (Willk.) P. Silva and Rozeira	Endangered endemic species	Cistaceae	3% sodium alginate + 100 mM $CaCl_2$ solution	Shoot tips	Coelho et al. (2014b)
Tylophora indica (Burm. f.) Merrill	Endangered medicinal plant	Asclepiadaceae	3% sodium alginate + 50–100- mM $CaCl_2{\cdot}2H_2O$	Nodal segments	Faisal and Anis (2007), Devendra et al. (2011), Gantait et al. (2017)
Withania coagulans (Stocks) Dunal	Endangered medicinal plant	Solanaceae	3.0% sodium alginate + 100 mM $CaCl_2{\cdot}2H_2O$	Micro-cutting, viz., shoot tips and nodal segments	Rathore and Kheni (2015)

3.2 *Ceropegia spiralis* Wt. and *C. pusilla* Wt. and Arn.

Ceropegia spiralis Wt. and *C. pusilla* Wt. and Arn. which belong to Asclepiadaceae are endemic and endangered medicinal herbs. The tubers are edible, rich in starch, and also helpful as a nutritive stimulant (Nadkarni 1976; Reddy et al. 2006). Since it was in neck of being endangered and having prominent medicinal significance, conservation of these plants is an important strategy. There are few margins for in situ conservation of these species, like awfully low accessibility of seeds and reduced reproductive capability. In advance, the alternative techniques like synthetic seed production are quite required to conserve these plant species. The micropropagation of various species of *Ceropegia* has been studied via tissue culture, such as *C. juncea*, *C. spiralis*, *C. intermedia*, *C. hirsute*, and *C. sahyadrica* (Nikam et al. 2008; Karuppusamy et al. 2009; Murthy et al. 2010; Krishnareddy et al. 2011). *C. spiralis* plants were regenerated successfully from young nodal segments with 100% shooting frequency (Chavan et al. 2011). Micropropagation, microtuberization, and in vitro flowering protocol of *C. pusilla* are illustrated with maximum survival rate of explants (Kondamudi and Murthy 2011; Murthy 2011; Kalimuthu and Prabakaran 2013). Murthy et al. (2013) studied the efficacy of endangered *C. pusilla* and *C. spiralis* by encapsulating the explants with 2.5–3% sodium alginate and 50 mM $CaCl_2$ and examined the influence of composition of nutrients in alginate matrix on regrowth performance. In encapsulated *C. pusilla* and *C. spiralis* synthetic seeds, shoot tips show the highest percentage of regrowth frequency with 86.6 and 90.0%, respectively, in a MS medium with 3% sucrose, 3% sodium alginate, and BAP 3 mg/L. Further studies are needed for these plant species to meet the propagation supplies for reintroduction of these plants in natural environment.

3.3 *Dendrobium nobile Lindl.*

Dendrobium nobile Lindl. is an epiphytic orchid with therapeutic significance and native inhabitant to the states of China, Northeast India, Thailand, Nepal, and Myanmar. The gracious *D. nobile* is a member of the family Orchidaceae (Bhattacharyya and Kumaria 2015). The huge ethnobotanical, ornamental, and medicinal values of *D. nobile* have been focused to harsh anthropogenic pressure for centuries resulting the inhabitants fetching threatened and endangered in its natural environments (Faria and Illg 1995; Miyazawa et al. 1997; Ye et al. 2002; Yang et al. 2006). In the recent past, *D. nobile* has gained much biopharmaceutical and horticultural importance globally owing to its significant source of diverse secondary metabolites (Ng et al. 2012). Few reports are available on tissue culture aspects of *D. nobile*. Exploration on in vitro growth and rooting of *D. nobile* was illustrated by using different sucrose concentrations (Faria et al. 2004). Axillary buds of *D. nobile* var. Emma white orchid were micropropagated by using phytotechnology medium (Asghar et al. 2011). In vitro regeneration protocols of *D. nobile* were illustrated

with a high frequency of survival rate of 82–92% with significant genetic stability and higher antioxidant activity being reported (Malabadi et al. 2005; Bhattacharyya et al. 2016). Regarding synthetic seed concept, no sufficient research reports are available to meet the desired prerequisite. Cryopreservation of PLBs of *D. nobile* was demonstrated on the basis of encapsulation-vitrification and encapsulation-dehydration. Encapsulation-vitrification shows higher regrowth (75.9%) and survival rate (78.1%) compared to encapsulation-dehydration (53.3 and 50.2%), respectively. Successful adaptation of plantlets was noticed under ex vitro condition with 82% maximum survivability (Mohanty et al. 2012). Mohanty et al. (2013) demonstrated short-term storage of encapsulated PLBs of *D. nobile* using mannitol and sucrose as osmotica up to 60 days. PLBs were encapsulated in 3% sodium alginate solution and 100 mM $CaCl_2 \cdot 2H_2O$ solution, prepared in MS liquid medium with various osmotic concentrations (mannitol and sucrose) in a series of 0.0–15.0% (w/v). After storage, encapsulated synthetic seeds show successful regeneration on optimized regrowth medium consisting of half-strength MS medium with 2% sucrose and 0.6% agar together with 0.1 mg/L NAA and 1 mg/L BAP media for the regeneration PLBs (Mohanty et al. 2012). Highest survival frequency of stored encapsulated PLBs on regeneration medium was 78.20% which was achieved with 7.5% mannitol followed by 64.56% with 7.5% sucrose concentration. Conservation and storage of various valuable threatened, rare, and endangered species of orchid can be possible by adaptation of consecutive advance protocols like synthetic seed approach. To overcome and meet the commercial demand of this species, much more attention has to be focused to formulate eminent procedure to maintain and preserve the valuable germplasm of *D. nobile*.

3.4 Rotula aquatica Lour.

Rotula aquatica Lour. is a species of aromatic flowering shrub and included as a member of the family Boraginaceae. It is a rhoeophytic rare woody medicinal shrub native to India, dispersed in tropical Southeast Asia, Sri Lanka, and Latin America. The tubery root is the representative component as well as serves as a raw material for several significant ayurvedic drug procedures (Sivarajan and Balachandran 1994; Chithra et al. 2005). Plants were exploited by excavating the roots for their medicinal properties, causing them to endanger. In vitro regeneration methods for *R. aquatica* by rapid clonal propagation, through in vitro culture of mature nodal explants and further the rooted shoots, were effectively transmitted to field with 70–80% survival rate (Sebastian et al. 2002; Martin 2003b). Indirect somatic embryogenesis, encapsulation, and plant regeneration were examined with friable callus developed from internode and leaf explants. Cotyledonary embryos were encapsulated with sodium alginate 3% solution in half-strength $CaCl_2$-free MS basal medium with 3% sucrose and 50 mM $CaCl_2$ solution. It exhibits 100% conversion to plantlets on half-strength MS basal solid medium. Under field conditions, 95% of survival frequencies of the plantlets were established, and they were morphologically similar to the mother plant

(Chithra et al. 2005). For conservation of this valuable and rare medicinal plant, established protocol of encapsulation has to be developed further to open the windows of conservation.

3.5 *Spilanthes mauritiana (A.Rich. ex Pers.) DC.*

Spilanthes mauritiana (A.Rich. ex Pers.) DC., a monogeneric endangered herb, belongs to Asteraceae, originated from Eastern Africa, and is employed in the local pharmacoepia to treat mouth and throat infections (Watt and Brayer-Brandwijk 1962) and also as medication for diarrhea and stomach ache (Kokwaro 1976). Deficient in ethnobotanical facts and the limited availability of *S. mauritiana* have led to lack in the information of undifferentiated compounds of *S. mauritiana* (Bais et al. 2002). Several attempts have been made previously on in vitro regeneration of *Spilanthes* species, i.e., *S. mauritiana* through axillary buds (Bais et al. 2002) and *S. acmella* through hypocotyls, axillary bud, and leaf explants (Saritha et al. 2002; Haw and Keng 2003; Pandey and Agrawal 2009). The rooting is usually a very leisurely process in *S. mauritiana* and also few explants are inadequate to adopt the protocol for a large-scale plantation program. Large-scale propagation and conservation through synthetic seeds have been illustrated for *S. mauritiana* through encapsulation of axillary buds in 3% sodium alginate and 100 mM $CaCl_2{\cdot}2H_2O$. After 3 weeks of storage, all the synthetic seeds germinated and developed into plantlets on half-strength MS nutrient medium, and further retrieved synthetic seeds were hardened effectively with 90% survival frequency with normal morphological and growth behavior, but subsequent storage reduced developing capability (Sharma et al. 2009a). Additionally, protocol was developed for germplasm exchange and short-term storage of *S. mauritiana* by synthetic seeds of nodal segments via encapsulating with 4 and 3% sodium alginate and 100 mM $CaCl_2{\cdot}2H_2O$ with maximum conversion of 83% under in vitro regeneration on basal MS medium complemented with 0.5 μM IAA and 5.0 μM BA. Under ex vitro condition, the success of 90% conversion with 80% survivability was achieved (Sharma and Shahzad 2014).

3.6 *Swertia chirayita Roxb. ex Fleming. H. Karst.*

Swertia chirayita (Roxb. ex Fleming. H. Karst.) is commonly known as "Chiretta" and is a decisively endangered medicinal herb and a member of the family Gentianaceae with valuable medicinal importance (Clarke 1885; Kirtikar and Basu 1984). *Swertia chirayita* is additionally recognized by a range of forenames like Bhunimba, Anaryatikta, Kairata in Sanskrit, Chiratitka, Chiaravata in Urdu, Qasabuzzarirah in Arab and Farsi, Sekhagi in Burma, and Chirrato or Chiraita in Nepal (Joshi and Dhawan 2005). This ethnomedicinal herb is also renowned for its

typical bitter flavor, incentive by the existence of diverse chemical elements such as swerchirin, amarogentin, swertiamarin, and other bioactive amalgams that are instantly related with welfare of human health (Joshi and Dhawan 2005). The complete plant is broadly utilized by local group for the healing of inflammation, hepatitis, and digestive illness (Bhatt et al. 2006). Owing to its extreme over-misuse from the likely habitat, constricted geographic frequency (Bhat et al. 2013), unsolved intrinsic tribulations of seed germination and seed viability (Badola and Pal 2002; Joshi and Dhawan 2005), incessant deforestation, and inadequate efforts for its replacement, *S. chirayita* is becoming extinct. There are a good number of studies on micropropagation of *S. chirayita* (Wawrosch et al. 1999; Joshi and Dhawan 2007; Chaudhuri et al. 2007, 2008, 2009; Wang et al. 2009; Balaraju et al. 2009, 2011; Pant et al. 2010a, b; Jha et al. 2011; Kumar et al. 2014, 2018; Shailja et al. 2017). Seed germination rate and viability of *S. chirayita* are awfully poor. Kumar and Chandra (2014) demonstrated a production of synthetic seeds of *S. chirayita* and competent procedure for plant in vitro regeneration through somatic embryogenesis from in vivo leaf explants of *S. chirayita* with maximum germination frequency of 76% on MS medium with 0.5 mg/L of kinetin and 0.5 mg/L of 2,4-D. Synthetic seeds of *S. chirayita* were by encapsulation of torpedo stage embryos in 4% (W/V) sodium alginate gel and dropped into 100 mM $CaCl_2 \cdot 2H_2O$ solution. Synthetic seed shows maximum conversion frequency of 84% germination on MS medium included with 0.5 mg/L NAA and 1 mg/L BA. Under ex vitro condition, regenerants were productively acclimatized with 80% conversion frequency in soilrite and sand in 1:2 ratios. However, substitute advances for effective conservation and propagation are indeed necessary to evade the feasible annihilation of this significant plant species.

3.7 Tecomella undulata (Sm.) Seem.

Tecomella undulata (Sm.) Seem. belongs to the Bignoniaceae family and is well known by different names like honey tree, rohida, marwar teak, ammora, white cedar, and desert teak. It is economically important monotypic species native to India and Arabia (Randhawa and Mukhopadhyay 1986). The plant is renowned for its medicinal healing values in Ayurveda (Oudhia 2005). In periodic time, it has been utilized to heal spleen and liver disorders, inner tumors, conjunctivitis, abdominal illness, hepatosplenomegaly, wound remedial, as a blood purifier, plus to cure syphilis, gonorrhea and also gratifying in hepatitis (Singh and Gupta 2011; Chal et al. 2011). Significant studies have been overviewed on tissue culture studies of *T. undulata* from juvenile terminal and axillary explants (Arya and Shekhawat 1986) as well as adult tissues acquired from 10 to 15 years mature plant (Bhansali 1993), nodal sections obtained from older trees (Rathore et al. 1991; Bhansali 1993; Kumari and Singh 2012; Tyagi and Tomar 2013; Chhajer and Kalia 2017), cotyledonary nodes, seedling explants, hypocotyls, and root fragments (Nandwani et al. 1995, 1996; Aslam et al. 2006; Singh et al. 2009a; Varshney and Anis 2012). Synthetic seed regeneration approach was demonstrated in encapsulated nodal

segments of *T. undulata* obtained from mature tissues with 3% sodium alginate, synthesized in MS liquid medium and 100 mM $CaCl_2 \cdot 2H_2O$ including 2.5 μM BA and 0.5 μM IAA, and further successive conversion of encapsulated synthetic nodal segments into shoots was achieved with optimal regeneration rate of 96.50% on MS medium with 10.0 μM BA under in vitro conditions (Shaheen and Shahzad 2015). In advance, influence of silver nanoparticles (AgNPs) on ACS gene expression profile was examined in the in vitro regenerated leaves of *T. undulate* and proposed that AgNPs improve the lifespan of explants as well as increase multiplication frequency (Sarmast et al. 2015). For long-term conservation of *Tecomella undulate*, even supplementary work has to be focused on synthetic seed approach to overcome the endangered status and to reintroduce the plant back to its natural ecosystem.

3.8 Tuberaria major (Willk.) P. Silva and *Rozeira.*

Tuberaria major (Willk.) P. Silva and Rozeira. belongs to family Cistaceae. It is an endemic small perennial plant of the Algarve, in the south of Portugal. Globally, ecological and geographical occurrence is rare, because distribution of this plant species is constrained to a very petite region reliable to a particular territory at a provincial comparison range. The foremost risks to this species are the invariable pressure of human and rising urbanization in the province. According to European legislation, *T. major* is a precedence and endangered plant species, and its protection is provided by the European Habitats Directive 92/43/CEE and by Portuguese law (reference 140/99 from April 24; ICN (Instituto da Conservação da Natureza 2006). The legitimate defense entails with the conservation of ecosystems and biodiversity in their normal territories; however, it might not always be ample to conserve the genetic diversity of a specified species. Therefore, it is essential to unite some conservation plan that will balance and maintain each other. Studies on the cryopreservation of *T. major* seeds were previously carried out (Goncalves et al. 2009a), and a protocol for in vitro micropropagation has also been illustrated (Goncalves et al. 2010), indicating imperative task for the conservation of this species exterior to its innate habitat. Plantlets of *T. major* formed in vitro are used to appraise the viability in long-term conservation method via cryopreservation of the different plant material to seeds. Coelho et al. (2014b) demonstrated *T. major* shoot tips cryopreservation by two ways, i.e., encapsulation-dehydration and vitrification. They stated that maximum percentage of regrowth was observed in encapsulation-dehydration and vitrification method with 67 and 60% survival through 3% sodium alginate solution synthesized in medium of MS having sucrose, 0.35 M, and 100 mM $CaCl_2$ solution cultured on MS semisolid medium amended with 0.2 mg L^{-1} zeatin at 25 °C in the dark, respectively. The appropriate exposure times were found to be 60 min in plant vitrification solution and 3 h desiccation for encapsulation-dehydration. The practice of cryopreservation techniques confirmed to be a significant positive feature in the conservation of endangered plant species and will further harmonize the strategies of conservation once developed.

3.9 *Tylophora indica (Burm. f.) Merrill*

Tylophora indica (Burm f.) Merrill is an endangered woody perennial climber, resident to the hills (≤900 m a.s.l) and plains of Southern and Eastern India (Anonymous 2003) and usually recognized as "Antamul or Indian ipecac." Indian Ipecac is a petite, more pronged, slender, velvety, climbing, or twining herb by yellowish sap. Traditionally, this native remedial plant has been utilized by ethnic groups in confident provinces of India for the healing of rheumatic pain, dermatitis, bronchitis, and bronchial asthma. The main vibrant element of this plant is the alkaloid tylophorine, accountable for its strong anti-inflammatory act (Gopalakrishnan et al. 1980; Bentley and Trimen 1992; Jayanthi and Mandal 2001; Faisal and Anis 2003). The lack of appropriate farming practices, demolition of plant habitation and uncontrolled compilation of plants from their innate environments, has led to a quick decline of *T. indica* in its natural environment (Jayanthi and Mandal 2001). Previously, substantial attempt has been performed on in vitro regeneration of this medicinally important endangered plant species by means of different explants (Sharma and Chandel 1992; Faisal and Anis 2003; Chaudhuri et al. 2004, 2006; Faisal et al. 2005). Owing to synthetic seed production, the artificial seed development by alginate encapsulation basis became a proficient tool mutually for mass propagation as well as interim preservation of numerous medicinal plants having commercial significance (Gantait et al. 2015). Synthetic seeds of *T. indica* were developed through encapsulation of in vitro derived explants, like somatic embryos from leaf segments with 3% sodium alginate in MS solution and 50 mM $CaCl_2$. Conversion of plant from encapsulated somatic embryos shows 22.4% germination and 6.8% rejuvenation frequency into plantlets on MS half-strength medium (Devendra et al. 2011). Synthetic seeds were produced from nodal segments *T. indica* with 3% (w/v) sodium alginate and 100 mM $CaCl_2 \cdot 2H_2O$, and it confirms 91% maximum conversion frequency into plantlets in the MS medium comprising BA, 2.5 μM, and NAA, 0.5 μM, after 6 weeks of culture. Synthetic seeds of *T. indica* proposed by using calcium alginate beads also show successful 43% of conversion to plantlets, when they were sown directly into Soilrite™ moistened with 1/2MS salts after 6 weeks. Encapsulated nodal segments were further successfully acclimatized and hardened into plants in soil, with a conversion rate of 90% (Faisal and Anis 2007). Synthetic seeds by in vitro nodal segments [(4 ± 1) mm long] reveal successful encapsulation, resulting in sphere-shaped synthetic seeds of similar morphology through 75 mmol L^{-1} $CaCl_2 \cdot 2H_2O$ plus 3% sodium alginate with 93.3% conversion frequency. The initial conversion of synthetic seeds within 7 days of incubation occurred in half-strength liquid MS medium. After 30 days of storage period at 15 ± 1 °C, 70% of conversion frequency was recorded without further decline even in subsequent 45 days of storage (Gantait et al. 2017), and their potentiality was determined at ambient temperature for storing of artificial seeds ensuring maximum post-storage.

3.10 *Withania coagulans (Stock) Dunal.*

Withania coagulans Dunal. belongs to the family Solanaceae and is generally called as "Indian cheese maker" or "vegetable rennet" since fruits and leaves of this plant are used as a coagulant and are also one of the vital medicinal plants. Withanolides isolated from *W. coagulans* aqueous fruit extract comprise cardioprotective, hepatoprotective, and anti-inflammatory activity (Budhiraja et al. 1983, 1986). Due to overexploitation this plant is considered as highly endangered. Significant research was carried out on in vitro regeneration in *W. coagulans* by using different explants (Rathore et al. 2012, 2016; Purushotham et al. 2015; Joshi et al. 2016). Rathore and Kheni (2015) illustrated synthetic seed production of *W. coagulans* micro-cuttings by alginate encapsulation with 3% sodium alginate and 100 mM of $CaCl_2 \cdot 2H_2O$ with maximum 96% of regrowth on regeneration medium, i.e., 0.75% MS agar-gelled medium supplemented 0.57 μM of IAA and 1.11 μM of BAP. They demonstrated efficiency of in vitro plantlet regeneration for rapid multiplication, storage for a short term, and also distribution of germplasm. After the 60-day storage period at 4 °C under sterile environment, the encapsulated explants showed 72% revival of plantlets on regeneration medium. The derived synthetic seed plantlets were effectively established and acclimatized. Regarding work on *W. coagulans* synthetic seeds is still lacking, and much emphasis is needed for long-term conservation and restoration of germplasm by encapsulation techniques.

4 Ornamentally Important Endangered Plant Species Used for Synthetic Seed Production (Table 3)

4.1 *Buxus hyrcana Pojark.*

Buxus is a genus which belongs to the Buxaceae family. *Buxus hyrcana* is commonly used for landscape and gardens. Cuttings of *B. hyrcana* are frequently used for production of softwood, semi-hardwood, and hardwood (Orhan et al. 2012). *B. hyrcana* is an ornamental shrub beneath threat of annihilation. Due to invasion of pathogens, this flowering shrub is a crucially endangered species. Earlier studies illustrate various attributes and health benefits of *B. hyrcana* including antioxidant and anti-inflammatory deeds activity, owing to its alkaloids and triterpenoids compounds (Babar et al. 2006; Choudhary et al. 2006; Esmaeili et al. 2009; Ata et al. 2010; Ebrahimzadeh et al. 2010; Mesaik et al. 2010). Micropropagation along with cryopreservation by encapsulation-dehydration method of *B. hyrcana* has been illustrated with 3% sodium alginate formed in MS salt solution with 0.75 M concentration of sucrose and 100 mM $CaCl_2 \cdot 2H_2O$ solution. Significant regrowth (60%) was observed in cryopreserved shoot tip after storage of 3 months in MS medium included with 1.50 mg L^{-1} NAA and 0.50 mg L^{-1} BAP, whereas there is no survival pace noticed in non-encapsulated shoot tip on regeneration medium after storage

Table 3 List of ornamentally important endangered plant species used for synthetic seed production

Plant species	Importance	Family	Synthetic medium	Explant source	References
Buxus hyrcana Pojark.	Endangered ornamental shrub	Buxaceae	3% sodium alginate + 100 mM $CaCl_2 \cdot 2H_2O$ solution	Shoot tips	Kaviani and Negahdar (2017)
Cymbidium aloifolium (L.) Sw.	Endangered orchid with medicinal and ornamental importance	Orchidaceae	4% sodium alginate + 0.2 M $CaCl_2 \cdot 2H_2O$ solution	Protocorms	Pradhan et al. (2014) Pradhan et al. (2016)
Ipsea malabarica (Reichb. f.) J. D. Hook.	Endemic and endangered orchid	Orchidaceae	3% sodium alginate + 0.7% $CaCl_2$ solution	In vitro formed bulbs	Martin (2003a)
Vanda coerulea Grifft. ex. Lindl.	Endangered orchid	Orchidaceae	3% sodium alginate + 100 mM $CaCl_2 \cdot 2H_2O$ solution	PLBs	Sarmah et al. (2010)
Vanilla planifolia Jacks. ex Andrews	Endangered orchid with commercial sigificance	Orchidaceae	4% sodium alginate + $CaCl_2 \cdot 2H_2O$ solution	Shoot buds and protocorms	Divakaran et al. (2006)

(Kaviani and Negahdar 2017). Presently, there is a vast necessity to expand a stratagem for long-term preservation of *B. hyrcana* elite germplasm. However, still there is no relevant approach in concern to preserve this threatened species. Hence, more prominence is needed to research on in vitro propagation and synthetic seed concept to develop the authentic procedures to maintain the *B. hyrcana* germplasm and also for long-term conservation to re-establish the species in its natural environment.

4.2 Cymbidium aloifolium (L.) Sw.

The species of *Cymbidium*, originally depicted in 1703 as *Epidendrum aloifolium.* Further, Swartz in 1799 separated into a new genus, *Cymbidium*. *Cymbidium aloifolium* (L.) Sw. is an epiphytic threatened orchid of Nepal with significant medicinal and ornamental importance (Das et al. 2008; Pant and Raskoti 2013). *Cymbidium aloifolium* also reside in an important place in the everyday life of tribal people of Northeastern India owing to its medicinal and ornamental significances. The indigenous people mainly in hilly regions take huge pride in treasuring this plant because of its high usefulness in traditional therapeutic and cures and floriculture deal (Medhi and Chakrabarti 2009). Apart from its medical magnitude, this orchid also magnetizes the floriculture marketplace, by its long-lasting extremely dazzling beautiful flowers. Due to its diverse application, public evacuated this plant from its native wild habitat and thus attain to the group of threatened species (Raskoti 2009; Nongdam and Chongtham 2011; Pant and Raskoti 2013). *Cymbidium aloifolium* is at present lying on the edge of extinction from its normal habitat owing to overexploitation for trade market, deforestation, and lack of pollinators due to arbitrary application of pesticides and further variations of the biome (Chugh et al. 2009). Therefore, a distinctive propagation advance is needed to safeguard this economically significant, threatened orchid. Micropropagation via tissue culture practices has been broadly employed for the in vitro large-scale multiplication for various economically significant orchids. A choice of work has been described about in vitro development of *C. aloifolum* by using various explants (Nayak et al. 1997; Hossain et al. 2009; Nongdam and Chongtham 2011; Pradhan et al. 2013; Trunjaruen and Taratima 2018). Synthetic seed technology with its beneficial advantage for germplasm storage has a huge potential for large-scale propagation loom of *C. aloifolium* ensuing field-ready propagules. *Cymbidium aloifolium* was studied for the development of synthetic seeds by encapsulation of 3-week-old protocorm with 4% sodium alginate and 0.2 M $CaCl_2 \cdot 2H_2O$ solution. This encapsulation results in the regeneration of 85% plantlets on MS media and was successfully hardened (Pradhan et al. 2014, 2016). Synthetic seed germination by asymbiotic method using protocorms offers a helpful mode to restore the plants in the native habitat, for germplasm conservation, as well as for commercial proliferation. Haphazard collection for illegal trade, habitat loss, and growing requirement are main reducing factors for distribution of this orchid species in its native residents

(Pradhan et al. 2013, 2016). However, modest is known about the synthetic seed path of *C. aloifolium*. Further, development of sequential procedure and paradigm will help substantially to overcome the extinction of this species.

4.3 Ipsea malabarica (Reichb. f.) J. D. Hook.

Ipsea malabarica (Reichb. f.) J. D. Hook. is an endangered and endemic rhizomatous terrestrial orchid of the Kerala Western Ghats, and they are generally recognized as "the Malabar Daffodil Orchid." The genus *Ipsea* is endemic to India in high-altitude hills of the southern Western Ghats and Sri Lanka. Earlier, it was believed that this species was extinct, and after a period of 132 years, i.e., in 1982, this species was further rediscovered (Manilal and Kumar 1983). It is endangered, and endeavors have been made to propagate the species through tissue culture to reintroduce them into the native place (Kumar and Manilal 1987; Martin and Madassery 2005). *Ipsea* is commonly propagated by seeds and vegetative propagation by rhizomes. The germination rate pace of seeds is extremely poor. Furthermore, propagation of seed depends on the association of obligatory mycorrhiza, and due to cross-pollination, the progenies are not true to type. The rate of propagation of this species has a straight persuade on its path of extinction. Poor propagation pace along with habitat loss along the Kerala Western Ghats has thrusted this terrestrial orchid to the category of endangered. Vegetative propagation by rhizomes is sluggish and not enough to overcome the risk of extinction. Propagation of this endemic and endangered orchid has been described in vitro (Gangaprasad et al. 1995; Martin and Pradeep 2003; Martin and Madassery 2005). Clonal propagation and synthetic seed approach by encapsulation of in vitro formed bulbs of *I. malabarica* were examined for its reintroduction to the innate haunt and facilitated with 100% conversion. In vitro formed bulbs were encapsulated with 3% sodium alginate and 0.7% $CaCl_2$. Encapsulated in vitro formed bulbs aided 100% conversion, when cultured on hormone-free half-strength MS and 6.97 μM kinetin-supplemented medium. Additionally, as a stride to in situ conservation, 50 plantlets were reintroduced into their native territory, i.e., at Vellarimala (at 1300 m height) of the Kerala Western Ghats, and plantlets were flowered normally. Primarily, from a single explant within 250 days, development of an above 40,000 plantlets is possible. This established protocol assists significantly for this endemic endangered orchid and also stands confidently to hold back the threat of extinction (Martin 2003a). Encapsulated synthetic bulbs can serve as a potential loom for in vitro germplasm conservation of *I. malabarica*, as studied in various endemic and endangered species of orchids like *Renanthera imschootiana* (Chetia et al. 1998) and *Geodorum densiflorum* (Datta et al. 1999). Except above preliminary approach, there is no inclusive study on reinhabitation on this endemic and endangered species. Further, footsteps are needed to restore *I. malabarica* into their indigenous native consigns in nature.

4.4 *Vanda coerulea Grifft. ex. Lindl.*

Vanda coerulea is commonly called as blue orchid (Plants Database 2015), blue vanda, or autumn lady's tresses. It has been recorded in different countries like India (Assam, Arunachal Pradesh, Meghalaya, and Nagaland), Burma, Nepal, Northern Thailand, and Southern China (Yunnan) and also probably found in Bhutan, Laos, and Vietnam. It is recognized as Vandaar in Sanskrit and Kwaklei in Manipuri. The juice of the flower is applied as eye drops against glaucoma, cataract, and blindness. The sap extracted from its leaves is utilized to treat dysentery, diarrhea, and dermal disorders (Nadkarni 1954). In India, overexploitation and demolition from its habitat are the main threatening factors for its survivability (Pradhan 1985). Few attempts have been made for rapid and efficient outline for in vitro propagation of *Vanda coerulea* (Seeni and Latha 2000; Manners et al. 2010; Roy et al. 2011; Hrahsel and Thangjam 2015). The production of synthetic seeds in orchids is very helpful, as orchids form non-endospermic and tiny seeds. In regard to synthetic seed approach, germination frequency of *V. coerulea* was examined by encapsulating 40-day-old PLBs in sodium alginate and preserved for 120 days at 4 °C, and it confirms 72% significant rate of regermination. Further, encapsulated beads stored at room temperature results in 50% germination in MS media. Conversely, unencapsulated PLBs preserved at room temperature and for 30 days at 4 °C did not show any germination (Devi et al. 2000). *V. coerulea* protocorms were productively cryopreserved by encapsulation-dehydration, and it shows highest regrowth of 40% amid with 35% water content subsequent to 8 h of dehydration with no significant morphological difference between non-cryopreserved and cryopreserved plantlets (Jitsopakul et al. 2008). Plant regeneration, short-term storage, and alginate encapsulation from PLBs of Aranda Wan Chark Kuan "Blue" × *Vanda coerulea* (a monopdial orchid hybrid) and conversion into completed plantlet regeneration were demonstrated. They recorded maximum percent of germination even after being stored for 180 days at 25 °C with 76.9 and 70.2% germination and conversion frequency and further acclimatized successfully with 92% of survival rate (Gantait et al. 2012). Encapsulation method of *V. coerulea* with 3% sodium alignate and 100 M $CaCl_2 \cdot 2H_2O$ was developed by PLBs obtained from leaf explants as an alternative tool for conservation. Optimal duration of storage for germination of encapsulated beads of *V. coerulea* was examined. Encapsulated PLBs show significant percent of germination (94.9%) in Ichihashi and Yamashita basal medium, and additionally encapsulated PLBs retain their viability up to 100 days at 4 °C (Sarmah et al. 2010). The above findings suggest that the synthetic seed approach confirms to be the most useful alternative tool for conservation of this endangered species.

4.5 *Vanilla planifolia Jacks. ex Andrews.*

Vanilla planifolia is an endangered vanilla orchid species, which belongs to the family Orchidaceae. *Vanilla planifolia* is a crop of immense commercial significance

as the source of natural vanillin, the main component of flavor industry (Divakaran et al. 2006). It is inhabitant to Mexico, Central America, the Caribbean, and Northeastern South America. Commercially, vanilla is grown for its pods, from which the flavoring substance, vanillin, has been extracted, and this is the only orchid with edible fruits (Geetha and Sudheer 2000). Compared to other vanilla-producing countries, Mexico is the center of origin of vanilla and has the largest genetic diversity of *V. planifolia*. However, currently wild inhabitants of vanilla are threatened with extinction. Vanilla is propagated exclusively through asexual propagation (cuttings), but stem cutting collection from plants growing in the wild results in the detained growth and development of the mother plants (Lubinsky 2003). To preserve the genetic resources of vanilla and for broadcasting uniform and disease-free elite material to growers, in vitro micropropagation by synthetic seed method is an effective approach. Initial exertion on in vitro propagation of vanilla is referred by Knudson, which was carried out in 1947 by Bouriquet with germinating seeds (Knudson 1950). Propagation of *V. planifolia* in vitro has been studied extensively using various propagules, explants, and culture medium, viz., through callus (Janarthanam and Seshadri 2008), protocorms, root tips (Philip and Nainar 1986), nodal explants, shoot tips, (Giridhar et al. 2001; Oliveira et al. 2013; Morwal et al. 2015), and axillary bud explants (Giridhar and Ravishankar 2004; Lee-Espinosa et al. 2008). Recently, an advance approach like hormetic and antimicrobial significances of silver nanoparticles were examined on in vitro regeneration of *Vanilla planifolia* by a temporary immersion method (Spinoso-Castillo et al. 2017). Synthetic seed development was demonstrated for in vitro regenerated shoot buds and protocorms by encapsulation with 4% sodium alginate and $CaCl_2 \cdot 2H_2O$ solution with 80% germination rate. Further, they standardized on slow growth medium that shoot cultures could be stored for an above 7 years with annual subculture. Further, in vitro preserved material is recovered and multiplied into normal plants in MS medium supplemented 0.5 mg L^{-1} IBA and 1.0 mg L^{-1} BA (Divakaran et al. 2006). Further, cryopreservation of apices from in vitro grown-up plants of *Vanilla planifolia* using three different techniques, i.e., encapsulation-dehydration, vitrification, and droplet-vitrification, was examined. Maximum survival frequency of 30% with regeneration rate of 10% of new-fangled multiple shoots was attained by the droplet-vitrification procedure with experimental parameters of 1-d preculture of apices with 0.3 M sucrose on solid medium, consignment with 0.4 M sucrose plus 2 M glycerol solution for 20–30 min, and exposure to plant vitrification solution (Gonzalez-Arnao et al. 2009). Additional research for the cryogenic practice needs to be optimized to progress for successful report of cryopreservation of vanilla apices. From the above reports, it conveys that in vitro conservation approach by synthetic seeds is the best path for maintenance and exchange of vanilla genetic resources.

5 Rare Plant Species Used for Synthetic Seed Production (Table 4)

5.1 *Ansellia africana Lindl.*

Ansellia africana Lindl. is an important orchid, which belongs to the family Orchidaceae, and it is commonly known as leopard orchid. It is generally distributed in the subtropical regions of Southern Africa (Bhattacharyya and Van Staden 2016). Owing to its significant medicinal practice and commercialization of *A. africana*, it has resulted in increased collection from wild inhabitants. The risk condition of *A. africana* has been classified as "vulnerable" by IUCN and by the Red List of South African Plants (2015) (http://www.iucnredlist.org/details/44392142/0). Together with the effect of overexploitation, *A. africana* has an extremely slow seed germination rate, i.e., below 3%, and also needs successful mutualistic fungal involvement for the seedlings to survive (Vasudevan and Van Staden 2010; Papenfus et al. 2016). This particular orchid has been utilized by the various African tribes and traditionally used in the healing of madness. Current research studies illustrate that *A. africana* roots have effective anti-acetyl cholinesterase action and are used in the treatment of nerve disorders, usually a path which may endow a solution for Alzheimer's disease (Chinsamy et al. 2011, 2014; Bhattacharyya and Van Staden 2016). Assessment of micropropagated *A. africana* has been performed by using different explants under in vitro conditions to know its genetic stability and influence different biotic and abiotic growth regulators (Zobolo 2010; Vasudevan and Van Staden 2011; Bhattacharyya et al. 2017). Even though few protocols have been reported on micropropagation of *A. africana*, still there is a dearth to meet the frequency of commercial relevance (Bhattacharyya and Van Staden 2016). Synthetic seed of *A. africana* has been developed by using PLBs obtained from nodal segments of seedlings with 3% sodium alginate and 100 mM $CaCl_2 \cdot H_2O$. Encapsulated sodium alginate beads show maximum response with 88.21% on medium supplemented with 7.5 μM *meta*-methoxy topolin 9-tetrahydropyran-2-yl (*mem*TTHP) (Bhattacharyya et al. 2018). Till date, there is no protocol that has been developed for long-term preservation illustrating the restoration of species to its natural habitat. To meet the economic necessity of this vulnerable orchid, relevant program and procedures have to be developed, and indeed research support has to be spotlighted to overcome the threat episode of *A. africana.*

5.2 *Ceropegia bulbosa Roxb. var.* bulbosa

Ceropegia bulbosa Roxb. var. *bulbosa* is distributed in the subcontinent parts of India (Punjab, Rajasthan, Western Ghats, Madhya Pradesh, etc.) (Murthy et al. 2012). *Ceropegia bulbosa* belongs to the Asclepiadaceae family, commonly identified as Khedula, Khadula, or Hedula. It is a significant medicinally vital herbaceous plant allocated throughout India (Kirtikar and Basu 1975; Jain and Defillips 1991)

Table 4 List of rare plant species used for synthetic seed production

Plant species	Importance	Family	Synthetic medium	Explant source	References
Ansellia africana Lindl.	Medicinally and commercially important vulnerable orchid	Orchidaceae	3% sodium alginate and 100 mM $CaCl_2 \cdot H_2O$	PLBs	Bhattacharyya et al. (2018)
Ceropegia bulbosa Roxb. var. *bulbosa*	Pharmaceutically important threatened plant species	*Apocynaceae*	3.0% Sodium alginate + 100 mM $CaCl_2$	Nodal segments	Dhir and Shekhawat (2013)
Cleisostoma arietinum (Rchb.f.) Garay	Rare Thai orchid species	Orchidaceae	3% sodium alignate + 100 mM $CaCl_2$	PLBs	Maneerattanarungroj et al. (2007)
Flickingeria nodosa (Dalz.) Seidenf.	Medicinally important vulnerable orchid	Orchidaceae	2% sodium alignate + 100 mM $CaCl_2$ solution	PLBs	Nagananda et al. (2011)
Serapias vomeracea (Burm.f.) Briq.	Threatened with extinction	Orchidaceae	3% Sodium alginate solution + 75 mM $CaCl_2$	PLBs	Bektas and Sokmen (2016)
Spathoglottis plicata Blume	Economically important vulnerable orchid	Orchidaceae	3.0% sodium alginate + 3.0% $Ca(NO_3)_2$ solution	PLBs	Haque and Ghosh (2017)
Spilanthes acmella (L.) Murr.	Threatened medicinal and pesticidal plant species	Asteraceae	3–4% sodium alginate and 100 mM $CaCl_2 \cdot 2H_2O$	Nodal segments, shoot tips	Sharma et al. (2009b) Singh et al. (2009b) Geetha et al. (2009)
Urginea altissima (L.f.) Baker	Threatened medicinal plant	Hyacinthaceae	3% sodium alginate and 100 mM $CaCl_2$	Shoot tips	Baskaran et al. (2018)

and possesses characteristic tuberous roots (Mabberley 1987). But here, in the current scenario, this plant is hardly ever observed due to critical compilation, habitat humiliation, reduced viability of seed and poor germination rate. The species has become threatened, due to overexploitation of the species from the native habitat and its rapid disappearance leading to its depletion in population. Tissue culture approach of plants tenders the likelihood for sustainable maintenance and reasonable utilization of biodiversity (Shekhawat et al. 2002; Mathur et al. 2002a, b, 2008; Jana and Shekhawat 2011), and it can be used for the ecorestoration of the plant. Micropropagation of *C. bulbosa* was described by a few researchers (Patil 1998; Britto et al. 2003; Goyal and Bhadauria 2006; Rathore et al. 2010) using nodal explants and epicotyl explants (Phulwaria et al. 2013). Furthermore, these conservative modes of propagation cannot accomplish the demand of pharmaceutical industries. Synthetic seeds were attempted by encapsulation of *C. bulbosa* var. *bulbosa* by using the segments of nodal with 3% sodium alginate and 100 mM $CaCl_2 \cdot 2H_2O$. Significant percentage of conversion frequency of the encapsulated nodal segments into plantlets was accomplished on MS medium complemented with BA at 8.88 μM concentration, resulting in 100% conversion of encapsulated nodal segments into shoots and can be effectively preserved up to 60 days at lower temperature of 4 °C with a 50.7% survival rate of recurrence (Dhir and Shekhawat 2013). Accordingly, in future there is a need for the development of even more reliable protocol for in vitro synthetic seed restoration way for the protection and conservation of *C. bulbosa* var. *bulbosa.*

5.3 *Cleisostoma areitinum (Rchb.f.) Garay.*

Cleisostoma areitinum (Rchb.f.) Garay. is a rare Thai orchid species. Orchid species are very well recognized as imperative ornamental plants in Thailand, particularly wild orchids. *Cleisostoma arietinum* is an epiphytic orchid and native habitat of Thailand. Also, it is quite often found in Southeast Asia (Northeast India, Burma, Thailand, Laos, Malaysia, and Vietnam). This particular orchid is in the stage of vulnerability as it is very rare to be noticed due to improper maintenance, risk of losing progressions due to contamination, difficulty in maintenance under in vitro collection, methodology errors, or somaclonal variations. Till date, there are no reports related to in vitro propagation of *C. areitinum*, and regarding synthetic seed method. Also, the development of sufficient standard protocol is quite lacking. In this regard, a preliminary cryopreservation of *C. areitinum* was examined through encapsulation-dehydration method by using protocorms. Protocorm explants were immersed in a solution of 3% alginate prepared with new Dogashima medium and then dropped into 100 mM $CaCl_2$ solution. Cryopreserved explants show significant regrowth percentage of survival on new Dogashima semisolid medium with 0.25 M sucrose for 1 week in dark conditions. Cryopreserved protocorms show 49% of survival rate and viability of protocorm confirms 77% by 2, 3, 5-TTC assay after storage of 2 weeks. This approach relies in simple, reliable protocol for *C. areitium*

cryopreservation by encapsulation (Maneerattanarungroj et al. 2007). Presently, there is a gigantic importance to further channelize and safeguard the germplasm of *C. areitium* by developing standard protocols for conservation stratagem.

5.4 *Flickingeria nodosa (Dalz.) Seidenf.*

Flickingeria nodosa (Dalz.) Seidenf. is a epiphytic medicinally important orchid with pseudobulbs, noticed in Sri Lanka, Eastern Himalayas, and in different divisions of Western Ghats of India, namely, Hassan, Kodagu, Udumbansholai, and Uttara Kannada (Abraham 1981; Rao 1998). Previously, it has been renowned as *Ephemerantha macraei* (Lindl.) (Rao 1998). Successively, it starts disappearing from its normal habitation rapidly due to over-misuse by humans and results in the status of vulnerable species (Kumar et al. 2001). It has immense medicinal significance in traditional system of medicine (Gupta et al. 1970; Nagananda and Satishchandra 2013). In "Charak Samhita," it is well known as Jeewanti which means life promoter. Preliminary in vitro regeneration protocol was studied on *F. nodosa* via asymbiotic seed germination with five different basal media. *F. nodosa* shows germination only on Lindemann orchid medium with 68% germination frequency (Wesley et al. 2013). The foremost phytoconstituents of *F. nodosa* are alkaloid Jibentine and α- and β-jivantic acids (Gupta et al. 1970). In the environment, orchids usually grow via seeds, but in the nonexistence of mycorrhizal alliance; additionally they don't grow in adequate quantity, hence its relies as a vulnerable species form. These barriers can be defeated by implementing in vitro *propagation* of synthetic seed technique. Synthetic seeds or beads for *Flickingeria nodosa* were developed by ex situ germplasm conservation through encapsulation of PLBs with 2% sodium alginate dropped in 100 mM $CaCl_2$ solution; it shows 95% germination even after being stored at 4 °C for 3 months on 1X Burgeff's N3F medium, 2% sucrose prepared with adenine sulfate at 2 mg L^{-1} and 1 mg L^{-1} of IAA. Plantlets were successfully acclimatized and hardened with 85% survival frequency under indoor conditions (Nagananda et al. 2011).

5.5 *Serapias vomeracea (Burm.f.) Briq.*

Serapias vomeracea is an entomophilous plant and is a species of orchid group in the genus *Serapias*, but it cannot offer floral rewards to pollinators, as it does not generate nectar. *Serapias vomeracea* is commonly known as long-lipped serapias or the plow-share serapias. The species is prevalent from south-central Europe, the Mediterranean Basin to the Caucasus (Pignatti 1982; Delforge 1995; Rossi 2002; GIROS 2009). Due to their economic importance, excessive and uncontrolled collection of these orchid tubers and assorted imperative parts of the plant before the seed maturation lowers the limited inhabitants of vital species and further even

constrains few species to near extinction. Synthetic seed production, in vitro seed germination, tuberization, and plantlet growth of *Serapias vomeracea* have been demonstrated. Tissue culture approach of seed germination and development of *S. vomeracea* were illustrated via asymbiotic germination under in vitro conditions. The maximum germination rate of 84.03% was achieved on Orchimax plus activated charcoal medium complemented with BA at 2.0 mg/L. Micropropagation by synthetic seed formation with 3% sodium alginate and 75 mM $CaCl_2$ and its germination frequency and acclimatization to ambient conditions were examined by using PLBs of *S. vomeracea*. The germination frequency of synthetic seeds to seedlings was 100% on Orchimax medium including activated charcoal and 60% on peat substrate (Bektas and Sokmen 2016). *Serapias vomeracea* is evaluated as least concern, owing to the lack of proper micropropagation practices, deficient in synthetic seed process for mass scale fabrication and harming to their natural allocation caused by neighboring assembles. Henceforth, maximum emphasis has to be given to develop standard protocol and programs to conserve the elite *S. vomeracea* germplasm.

5.6 *Spathoglottis plicata Blume.*

Spathoglottis plicata Blume. is a terrestrial vulnerable orchid with horticultural importance bearing graceful flowers booming around the year but invasive in perceptive tropical habitats. *Spathoglottis plicata* generally known as the Philippine ground orchid belongs to the family Orchidaceae. It is native inhabitant of Southeastern Asia and is also observed in the Philippines, Australia, New Guinea, and in southwest Pacific Ocean Islands (Cribb and Tang 1982). A momentous number of orchids, like *Spathoglottis*, have been employed for industrial rationale and are gathered immensely from its natural territory which has led this group of plants likely to become endangered if trends continue. Seed germination and propagation of *S. plicata* is quite complex under in vitro conditions (Aewsakul et al. 2013). Few approaches on in vitro micropropagation have been reported previously (Kauth et al. 2008; Sinha et al. 2009; Novak and Whitehouse 2013; Hossain and Dey 2013), above of these in vitro propagation processes have trailed the straight path of restoration without any development of callus, and their regeneration rate of plantlet was reasonably low. Morphogenesis via callus formation by indirect pathway is the most effective way that can be used for in vitro mutagenesis, selection, and plant transformation (Sahijram and Bahadur 2015). Hence, there is indeed prerequisite of competent procedure for regeneration via the indirect path to safeguard this horticulturally significant orchid. Khor et al. (1998) developed a complex conservation of two-coat methods for the *S. plicata* protocorms and seed encapsulation, i.e., alginate-chitosan and alginate-gelatin. As a result, 40% of large protocorms (1.6–2.0 mm) and 54% of seeds were capable to withstand desiccation treatment for 6 h. But, viability factor of the diminutive protocorms (0.7–0.9 mm) was significantly decreased, if they were desiccated prior to encapsulation. Further, viability percentage of seeds and protocorms increased significantly by encapsulation after

desiccation. Recently, Haque and Ghosh (2017) demonstrated *S. plicata* plantlet production with high-frequency through indirect PLB formation using callus by employing *Aloe vera* gel as a complex organic supplement for in vitro culture of orchid. For encapsulation process, 3% of sodium alginate and 3% of $Ca(NO_3)_2$ show most appropriate form for the synthetic bead formation with good germination rates (86.7%). Even after storage period of 90 days at 4 °C, synthetic seeds show 66.7% germination capability. Plantlets reveal 86.6% rate of survival, and 76.3% of these transferred plants formed flowers within 12–15 months of field transmit. Till now, significant contribution was not contributed to maintain *S. plicata* plant. Thus, there is indeed a necessity to develop reliable protocol in the point of mass scale commercial propagation and also proconservation for a long-term of this imperative orchid.

5.7 *Spilanthes acmella (L.) Murr.*

Spilanthes acmella (L.) Murr. belongs to the family Asteraceae, a threatened medicinal plant that develops in tropics and subtropics. This plant species has been generally utilized in a folk therapy, e.g., for rheumatic, fever, and toothache (Haw and Keng 2003; Wongsawatkul et al. 2008), also consumed as fresh vegetable (Tiwari et al. 2011) and spices for Japanese appetizer (Leng et al. 2011). *Spilanthes acmella* refers to the significant medicinal plant disseminated in the tropical and subtropical provinces around the world with wealthy source of beneficial and medicinal components. Over the precedent petite years, substantial attempts have been performed on in vitro propagation of this threatened plant species using different explants (Pandey and Agrawal 2009; Singh et al. 2009c; Yadav et al. 2012; Sharma and Shahzad 2013; Kurian and Thomas 2015; Joshi et al. 2015; Algabri and Pandhure 2017; Sana and Rani 2017). Few reports are available on synthetic seed concept of *S. acmella*. Synthetic seed production of *S. acmella* was reported previously using shoot tips with sodium alginate (3%) and $CaCl_2$ (100 mM). Significant conversion frequency into plantlets was achieved in the encapsulated shoot tips on full-strength liquid MS medium. Regenerated plantlets from encapsulated shoot tips were successfully ascertained under field environment with 90.0% survival rate. After storage for 60 days at low temperature (4 °C), encapsulated shoot tips show survival response was 50% (Singh et al. 2009b). Synthetic seeds were illustrated by nodal segments gained from in vitro raised seedlings of *S. acmella* using 4% sodium alginate and 100 mM $CaCl_2 \cdot 2H_2O$. Successful conversion of encapsulated nodal segments into plantlets was achieved at the rate of 87.8% on MS medium with α-NAA (0.5 μM) and BA (10 μM) after 6 weeks of culture. Regenerated plants from encapsulated synthetic nodal fragments were effectively toughened, adapted, and established in soil, with a survival frequency of 90% under ex vitro conditions (Sharma et al. 2009b). Further, morphogenic responses of synthetic seeds were studied in encapsulated shoot tips with 3% sodium alginate and 100 mM $CaCl_2 \cdot H_2O$ to different kinds of growth regulators in various concentrations of encapsulated shoot tips. Encapsulated shoot buds show maximum conversion response of 92% into plantlet on MS basal medium

with 1.0 mg/L BAP after 2–4 weeks of subculture and were stored at 4 °C, and it remained viable for 12 weeks of storage period (Geetha et al. 2009). In the future, optimization is quite required to the practice of *S. acmella* synseed approach for long-term in vitro conservation.

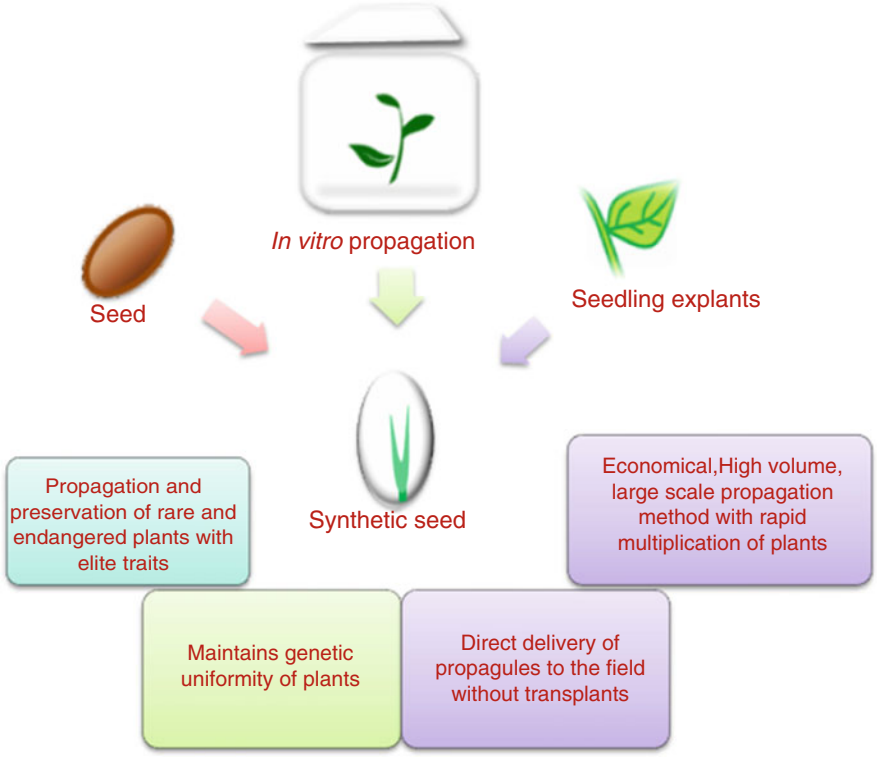
5.8 *Urginea altissima (L. f.) Baker.*

Numerous genera of Hyacinthaceae, including *Urginea*, contain ornamental and medicinal properties (McCartan and Van Staden 1999; SANBI 2017). Since they are ephemeral in nature, seeds of this genus should be sown immediately. *Urginea altissima* (L.f.) Baker is a bulbous perennial threatened medicinal plant (Williams et al. 2016). It is commonly identified as squills. Traditionally, this plant has been employed for the healing of asthma, influenza, warts, bronchitis, cancer, rheumatism, cardiac arrhythmia, and congestive heart failure (Watt and Brayer-Brandwijk 1962; Hutchings 1989; Hutchings et al. 1996; Langat et al. 2013). Due to demand for medicinal and ornamental applications, natural inhabitants of *U. altissima* are overexploited, thus resulted the plant to be cataloged as a threatened species in the Red Data Record of South African plants (Williams et al. 2016; SANBI 2017). Few in vitro propagation procedures have been illustrated for other species of *Urginea* (Jha et al. 1984; Jha and Sen 1986; El Grari and Backhaus 1987; Stojakowska 1993). However, for *U. altissima*, no significant proper assessment on in vitro propagation has been carried out. Synthetic seeds of *U. altissima* were produced by encapsulation of shoot tips, in which they examined adventitious shoot regeneration with 91.0%; 12.6 shoots per synthetic seed on MS semisolid medium amended with 10 μM metatopolin plus 2 μM of NAA after storage period of 15 days in dark condition at temperature of 25 ± 2 °C. Shoot tips of *U. altissima* were encapsulated with 3% sodium alginate prepared in MS liquid medium and 100 mM $CaCl_2$. In regrowth parameters, encapsulated seeds show adventitious shoot regeneration of 91.0%; 12.6 shoots per synthetic seed on semisolid MS medium supplemented with 10 μM mT and 2 μM NAA after 15 days of storage in darkness at 25 ± 2 °C. In the greenhouse all the plantlets were 100% successfully acclimatized (Baskaran et al. 2018). Further refined studies on in vitro propagation and synthetic seed production for *U. altissima* are needed to be focused to improve the conservation approach.

6 Future Perspectives

Synthetic seed technique is a boon for the vegetative propagation, preservation, and long-term conservation of elite germplasm of rare, endangered, and threatened species. In the present era, synthetic seed has immense applications in the field of agriculture with its strong aptitude of long-term storage and direct propagation of seedlings to field level. Plant species with precious elite germplasm with significant

commercial, medicinal values can be restored for future generations via this approach. Additionally, those plant species, which are not capable to produce seed, i.e., seedless species, can be able to propagate by means of synthetic seed approach. For the exchange of elite plant material from private and public laboratories and to transport across frontiers without spread of disease through aseptic path, development of synthetic seeds plays a major task by way of direct propagation behavior from nursery to field. The present scenario of synthetic seed to advance the innovation in agriculture zone still needs even more practical directed appliances. From the past few decades to till date, sustainable efforts have been made, but the relevance on application to conserve the elite germplasm and to restore them back to natural habitat is not yet channelized properly. Unfortunately, still there is a breakdown of efforts is being faced in concept of above norms. Direct sowing of synthetic seed in soil or in commercial substrates like compost, vermiculite, etc. is considered to be major limitations for the practical applicability of this technique. According to Murashige, synthetic seeds are an encapsulated distinct somatic embryo, a clonal product that can be utilized as true seed for storage, sowing, and for transport either in vitro or ex vitro conditions. Encapsulation coat of synthetic seed is measured as protective shield against drought and pathogens under different ecological factors, and thus they increase the shelf life of micropropagules. Now, it is well-established fact that synthetic seed approach is an efficient approach to carry elite germplasm in it. It also facilitates in polyploidy invention without any genetic recombination process; thus it holds up a major grip in plant breeding system. In transgenic plants, synthetic seed production via somatic embryos helps to carry single gene consigned in somatic cell and inherit to progenies successfully with same aptitude. In view of research measures, further study has to be carried out to progress non-embryogenic synthetic seeds to progress cultivation practices for its adaptation under unfavorable conditions. Even in-depth view is needed for long-term preservation of synthetic seed, and further priorities have to be oriented to overcome reduced survivability after storage period. As enlightened above in this chapter, flourishing endeavors were made by various researchers to recover and advance the eminence of few important elite plant germplasm with intervention of plant tissue culture and different synthetic seed methods. From all the above information, it proclaims that synthetic seed is a resourceful technology in propagation of plant. Synthetic seed technique opens up a new avenue for long-term conservation of precious privileged plant material and further facilitates to overcome extinction of endangered, rare, and threatened plant species.



Schematic illustration of synthetic seeds applications

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Application of Synthetic Seeds in Propagation, Storage, and Preservation of Asteraceae Plant Species

Dariusz Kulus

Abstract The artificial seed technology is one of the most important tools to plant scientist working with in vitro cultures. Encapsulation can provide a source of aseptic explant material that can be used if stock plants or in vitro cultures become infected with microorganisms. Moreover, the application of artificial seeds allows to maintain gene collections, which cannot be stored in liquid nitrogen. Up-to-date several studies on 13 Asteraceae genera were performed. It was found that synthetic seeds are suitable for medium-term storage under refrigeration conditions; however, long storage periods often lead to viability loss. The composition of the ideal gel matrix depends on the species and explant type. In general, however, the artificial endosperm should be supplemented with Murashige and Skoog (1962) salts and sucrose for in vitro germination. As for the recovery medium, the presence of benzyl adenine is beneficial. Also direct sowing of synseeds to ex vitro conditions is possible, provided that the artificial endosperm does not contain any organic compounds. The performed studies confirmed the stability of plants recovered from artificial seeds on the genetic, cytogenetic, biochemical, and phenotypic levels. Further research should be carried out to standardize optimum storage technique for better results and to prolong the artificial seeds viability.

Keywords Artificial seed · Synseed · Sodium alginate · Somatic embryo · Shoot tip · Callus

1 Introduction

The horticultural market is represented by numerous plant taxa. Those resources can be used in food production, medical, aromatic, and ornamental purposes. The Asteraceae is one of the most evolved, important, and diverse botanical families

D. Kulus (✉)
Faculty of Agriculture and Biotechnology, Department of Environmental Bases of Agriculture and Horticulture, Laboratory of Ornamental Plants and Vegetables Crops, UTP University of Science and Technology, Bydgoszcz, Poland

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distributed worldwide (Abraham and Thomas 2016). Nowadays, biotechnology-based methods are applied with the members of this family for various purposes (Teixeira da Silva and Kulus 2014). Synthetic seeds, i.e., artificially encapsulated plant tissues, can be utilized in the propagation, storage, and protection of the Asteraceae species. Numerous publications in this area are available.

The aim of this review was to summarize some information from the past decade on the application of artificial seeds with medicinal, ornamental, and threatened plant species of the Asteraceae family.

1.1 Asteraceae: Brief Description of the Family

The Asteraceae (previously Compositae) is a botanical family comprising 43 tribes, 1600–1700 genera, and about 24,000–30,000 species, which makes it one of the largest botanical families, representing approximately 10% of all flowering plants worldwide (Abraham and Thomas 2016). It is also known as sunflower, thistle, or daisy family. Its members exhibit a diversity of life forms, including annual, biennial, or perennial herbs, shrubs, trees, scramblers, as well as succulent and aquatic plants, which occupy almost every environment and continent except Antarctica (Bisht and Purohit 2010; Abraham and Thomas 2016). The Asteraceae includes ornamental, edible, noxious, medicinal, endangered, and invasive species (Achika et al. 2014). Many plants of this family are widely overexploited for various purposes (mainly folk medicine), and hence there is an urgent need for standardizing efficient propagation and preservation methods of genetic resources. The synthetic seed technology is a promising solution for both those issues.

1.2 Synthetic Seeds: Basic Concepts

The concept of synthetic seeds, also known as artificial seeds, manufactured seeds, or synseeds, was developed in the 1970s for potato (*Solanum tuberosum* L.) by a professor of biology and co-founder of the famous medium—Toshio Murashige (Murashige 1977). Synthetic seed consists of either a hydrated or dehydrated explant, naked or covered with a protective polymeric bead. The later approach is much more popular with Asteraceae. Primarily, only somatic embryos were encapsulated, but currently, the definition of artificial seeds is much wider (Gantait et al. 2015; Nic-Can et al. 2015). The biological material used for this purpose can be any organ or tissue that is able to reproduce the complete plant. This may include zygotic embryos, embryonic axes, shoot tips, axillary or adventitious buds, meristems, and even callus (Reddy et al. 2012). Explants are usually embedded in sodium alginate ($NaC_6H_7O_6$)—a polysaccharide composed of homopolymeric blocks of (1-4)-linked β-D-mannuronate and α-L-guluronate residues, extracted from the cell walls of brown algae thrown to the shores of the Atlantic. Because of nontoxic, inert, cheap, and

easily manipulable qualities, alginate is the most commonly applied polymer for the immobilization of plant cells (Sudarshana et al. 2013). In the presence of divalent cations, e.g., calcium, ion exchange occurs resulting in the alginate polymerization to a hard bead with rigid structure and large pore size (insoluble in water) that protects the explant (Kakita and Kamishima 2008). By adding nutrients, such as macro- and microelements, vitamins, etc., an artificial endosperm is formed that provides the explant with good conditions for growth, both in vitro and ex vitro. In the latter case, however, it is necessary to cover the synseed with an additional fungicidal layer, as the matrix is also a suitable medium for microbial contamination development.

Synthetic seeds combine the advantages of clonal and generative reproduction (Alatar and Faisal 2012). They facilitate the manipulation of explants and their short- and medium-term storage and transport and are used in large-scale production at low cost (Reddy et al. 2012). Due to their small size, 2–3 mm in diameter, artificial seeds can be stored in a Petri dish, vial, or other vessel, in minimum volume, i.e., 20–30 pieces, in a single tube. After adding the dehydration step (chemical and/or physical), synseeds are also used in cryopreservation, i.e., storage in liquid nitrogen at −196 °C (Kulus and Zalewska 2014a). An alginate-gelled matrix surrounding an explant provides the mechanical support needed to protect the tissue within encapsulation medium during long-term storage (Sujatha and Kumari 2008). This helps to preserve germplasm, which in turn plays an important role in the maintenance and management of plant genetic resources (Geetha and Gopal 2009; Kulus 2016).

2 Application of Synthetic Seed Technology with *Asteraceae* Family

Synthetic seeds have been applied with numerous Asteraceae plant species (Table 1). Nodal segments are the most often encapsulated explants, although shoot tips, axillary buds, or even whole microshoots are also used for this purpose. Only three protocols described the possibility of producing synseeds with somatic embryos, and one with callus.

The optimal sodium alginate and calcium chloride concentration for bead production, as well as germination medium composition, may change with different explant types in addition to different plant species. Moreover, differences in ideal sodium alginate concentration may also vary in terms of chemical commercial source, due to the difference in pore size of Na-alginate (Sharma and Shahzad 2014).

Table 1 Application of synthetic seed technology in various Asteraceae species

Plant material	Procedure	Germination	Remarks	References
Ajania pacifica 'Bengo' 7-day-old shoot tips, 2 mm in length	Single-node explants cultured on MS medium with 3% sucrose (7 days) → shoot tips coated in 3% Na-alg. (10 min) + MS salts +3% sucrose → polymerization in 100 mM $CaCl_2$ (30 min) → osmotic dehydration in 0.3–0.9 M sucrose (5 days) → air desiccation (4 h; 41% MC) → culture on MS medium with 1.11 μM BA or 1.16 μM KIN for 30 days at 25 ± 1 °C in light (16-h photoperiod)	70–90% survival 30% germination	The presence of the artificial matrix improved the quality of the shoots, but dehydration had a deleterious effect on their germination. Rooting was similar with the untreated control	Kulus and Abratowska (2017)
Artemisia vulgaris Embryos	Coating in 2% Na-alg. → polymerization in 75 mM $CaCl_2$ (30 min) → culture on MS medium with 4.33 μM GA_3 and 2.85 μM IAA and 40 $mg \cdot L^{-1}$ ascorbic acid for 30 days at 22 °C in dark	90.0%	Development of multiple shoots from a single synthetic seed was reported. The regenerated plantlets were transferred to sterile soil for hardening and acclimatization in the field	Sudarshana et al. (2013)
Nodal segments	Coating in 2% Na-alg. → polymerization in 50 mM $CaCl_2$ (20 min) → culture on MS medium with 4.9 μM 2iP for 0, 20, 40 and 60 weeks at 5 °C in dark	85.0%	Recovery after 1–– 3 months, depending on the length of the storage time. Nodal segments that were stored for 40 and 60 weeks grew slower than those not refrigerated or stored for 20 weeks	Sujatha and Kumari (2008)
Atractylodes macrocephala Axillary buds	Coating in 2.5% Na-alg. and 2.85 μM IAA → polymerization in 2% $CaCl_2$ (9 min) → culture at 25 °C in light (16-h photoperiod)	100% survival 82.0% germination	Germination of synthetic seeds with the addition of ½ MS salts into the matrix was similar to that of synseeds with the addition of distilled water	Wang et al. (2011)

(continued)

Table 1 (continued)

Plant material	Procedure	Germination	Remarks	References
Chrysanthemum × *grandiflorum* Shoot tips (non-disinfected)	Coating in 2.5% Na-alg. (1 min) → polymerization in 100 mM $CaCl_2$ (30 min) → sowing on vermiculite for 4 weeks at 20 ± 4 °C and 80% RH in natural light	70.0%	All used chemicals were non-autoclaved	Hung and Dung (2015)
Shoot tips	Coating in 3% Na-alg. (10 min) → polymerization in 100 mM $CaCl_2$ (30 min) → culture for 90 days at 24 ± 2 °C in light (16-h photoperiod)	82.5–97.5%	Presence of the bead inhibited spontaneous rooting, which is otherwise common in chrysanthemum cultured in vitro	Kulus and Zalewska (2014b)
Cineraria maritima Microshoots (3–5 mm in length)	Coating in 3% Na-alg. + ½ MS salts + 1.5% sucrose → polymerization in 3% $CaCl_2$ (25 min) → storage for 6 months at 25 ± 2 °C with SDW spraying in 15-day intervals → germination on semisolid MS medium	82.4%	A total of 90% plants survived acclimatization and transfer to the greenhouse	Srivstava et al. (2009)
Eclipta alba Shoot tips Nodal segments	Coating in 3% Na-alg. and 3% sucrose → polymerization in 1.11% $CaCl_2$ (30–45 min) → culture on MS medium for 8 weeks at 4 °C in reduced light (1.5 µmol m^{-2} s^{-1}) → recovery at 20 ± 2 °C, in light (18-h photoperiod; 30 µmol m^{-2} s^{-1})	82.6–85.4%	Growth of encapsulated explants found 5–8 days after transferring to recovery conditions with no PGRs Nearly 100% survival after acclimatization to ex vitro conditions was reported	Ray and Bhattacharya (2010)
Nodal segments	Coating in 3% Na-alg. → polymerization in 100 mM $CaCl_2$ (20–30 min) → storage on MS medium with 0.88 µM BA for 60 d	51.2%	Shoots and roots emerged from encapsulated explants after 1 and 2 weeks of culture, respectively Non-encapsulated	Singh et al. (2010)

(continued)

Table 1 (continued)

Plant material	Procedure	Germination	Remarks	References
	at 4 °C → recovery on MS medium with 0.88 μM BA		explant did not survive storage	
Gerbera jamesonii	Coating in 3% Na-alg. + 3% sucrose + 8.88 μM BA +2.69 μM NAA → polymerization in 100 mM $CaCl_2$ (30 min) → culture on MS medium for 30 days at 25 ± 1 °C, in light (16-h photoperiod)		The synthetic seeds could have been sawn directly to garden soil with 90 and 83% germination rates (microshoots and somatic embryos, respectively) The survival rate of plantlets reached 75% after transferring to the greenhouse	Taha et al. (2009)
Microshoots		75.0%		
Somatic embryos		63.2%		
Glossocardia bosvallea Embryoids	Embryogenic callus induced on leaves on MS medium with 7.77 μM BA +5.37 μM NAA → embryo coating in 2.5% Na-alg. → culture on MS medium for 25 days at 25 ± 2 °C	67.0%	Storage over 25 days decreased the viability of the encapsulated embryos, and no germination was reported from the capsules stored for 30 days	Geetha and Gopal (2009)
Hypochaeris radicata	Coating in 3% Na-alg. → polymerization in 35 mM $CaCl_2$ (30 min) → culture on MS medium with 8.88 μM BA for 4 months at 25 °C in light (16-h photoperiod)		Storage longer than 4 months decreased the regeneration ability of explants	Senguttuvan and Subramaniam (2014)
In vitro *leaf*		82.5%		
In vitro root		32.9%		
In vitro callus		51.1%		
Sphagneticola calendulacea Nodal segments	Coating in 2.5% Na-alg. (10 min) → polymerization in 75 mM $CaCl_2$ (30 min) → culture on semisolid ½ MS + 1.5% sucrose for 14 days at 25 ± 2 °C in light (16-h photoperiod)	84.4%	Acclimatization to ex vitro conditions on sand, soil, and vermicompost (1:1:1; w/w) with 89% survival frequency	Kundu et al. (2018)
Spilanthes acmella Shoot tips	Coating in 3% Na-alg. → polymerization in 100 mM	50.0%	The addition of MS nutrients into gel matrix improved	Singh et al. (2009)

(continued)

Table 1 (continued)

Plant material	Procedure	Germination	Remarks	References
	$CaCl_2$ (20–30 min) → culture on liquid MS medium for 60 days at 4 °C in dark		shoot and root emergence	
Shoot tips	Coating in 3% Na-alg. → polymerization in 100 mM $CaCl_2$ (30 min) → culture on MS medium + 4.44 μM BA for 4 weeks at 4 °C	92.0%	MS germination medium gave superior sprouting results followed by Nitsch and Nitsch (1969) and B5 media Storage over 4 weeks leads to a significant synseed viability loss with 60 and 20% germination rates after 10 and 12 weeks, respectively	Geetha et al. (2009)
Nodal segments	Coating in 4% Na-alg. → polymerization in 100 mM $CaCl_2$ (20–25 min) → culture on MS medium + 1 μM BA + 0.5 μM NAA for 6 weeks at 25 ± 2 °C in light (16-h photoperiod)	87.8%	Transfer to soil with 90% success rate	Sharma et al. (2009a)
Spilanthes mauritiana Nodal segments	Coating in 3% Na-alg. + MS salts + 3% sucrose → polymerization in 100 mM $CaCl_2$ (20 min) → storage in beakers moist with SDW for 4 weeks at 4 °C → germination on ½ MS medium	80.0%	Produced plantlets were covered with glass bottles and acclimatized for 4 weeks with 90% survival rate and no morphological alternations	Sharma et al. (2009b)
Nodal segments	Coating in 4% Na-alg. + MS salts + 3% sucrose → polymerization in 100 mM $CaCl_2$ (20–25 min) → germination on semi-solid MS medium + 1 μM BA + 0.5 μM	77.4%	Longer storage periods led to the conversion rate decrease (41% after 8 weeks) Explants coated with encapsulation matrix prepared in MS medium were more	Sharma and Shahzad (2014)

(continued)

Table 1 (continued)

Plant material	Procedure	Germination	Remarks	References
	IAA for 2 weeks at 4 °C		viable in comparison to water-based ones	
Stevia rebaudiana Nodal segments	Coating in 5% Na-alg. → polymerization in 50 mM $CaCl_2$ → culture on MS medium with 0.91 µM TDZ for 8 months at 25 °C	77.0%	87% acclimatization efficiency No morphological changes reported	Lata et al. (2014)
Nodal segments (0.2–0.5 cm in length)	Coating in 4% Na-alg. → polymerization in 75 mM $CaCl_2$ (30 min) → germination on MS medium for 8 days	60.0%	Acclimatization in soil with solarite and vermiculite (2:1:1) for 10–15 days with 60–70% survival rate	Khan et al. (2013)
Shoot tips	Coating in 3% sodium alginate → polymerization in 2.5% $CaCl_2$ → culture on MS medium + 8.88 µM BA + 4.92 µM IBA for 30 days at 4 °C in dark	70%	Conversion within 3 weeks. Acclimatization in peat and moss (1:1) substrate	Andlib et al. (2011)
Shoot tips	Coating in 4% Na-alg. → polymerization in 100 mM $Ca(NO_3)_2$ (30 min) →culture on MS medium + 1.11 µM BA for 5 weeks at 20 °C in dark	100%	The addition of osmotic agents into the culture medium was not an efficacious procedure	Nower (2014)
Taraxacum pieninicum Shoot tips	Coating in 3% Na-alg. → polymerization in 100 mM $CaCl_2$ (25 min) → culture on MS medium at 4 ± 1 °C in dark	96.7%	Non-stored (control) synthetic seeds were able to conversion into the whole plant in 46.7% The produced shoots were successfully rooted on the PGR-free MS medium and acclimatized to ex vitro conditions	Kamińska et al. (2018)

2iP 2-isopentenyladenine (6-(γ,γ-dimethylallylamino)purine), *B5* Gamborg et al. (1968) medium, *BA* benzyl adenine, GA_3 gibberellic acid, *IAA* indole-3-acetic acid, *KIN* N6-furfuryladenine (kinetin), *MC* moisture content, *MS* Murashige and Skoog (1962) medium, ½ *MS* half-strength MS, *NAA* 1-naphthaleneacetic acid, *Na-alg.* sodium alginate, *PGRs* plant growth regulators, *RH* relative humidity, *SDW* sterile distilled water, *TDZ* thidiazuron

2.1 *Ajania*

The *Ajania* genus is a novelty on the horticultural market, valued also in medicine. To date, only one paper focused on the possibility of producing synthetic seeds of *Ajania pacifica* (Nakai) Bremer et Humpries 'Bengo' for the propose of long-term storage (Kulus and Abratowska 2017). In this protocol, shoot tips were encapsulated in 3% calcium alginate, as described originally by Lynch in 2002. Next, the beads were dehydrated in sucrose gradient (0.3–0.9 M) for 5 days and then desiccated for 0–5 h (up to 39% initial moisture content). Afterwards, the synthetic seeds were inoculated on various recovery media.

Despite good survival of the synthetic seeds (70–90% after 30 days of culture), the stimulation of their further growth was problematic, regardless of the recovery medium, especially after dehydration (germination ~30%). Low dehydration tolerance is a serious limitant for long-term storage of biological diversity, as such material cannot be stored at sub-zero temperatures and has to be recovered quite often (Kulus and Zalewska 2014a). On the other hand, it was found that the presence of the alginate bead had a positive influence on the quality of the recovered plantlets, which had longer shoots and roots and a greater fresh weight as compared with the control (plantlets produced from naked shoot tips). This could be a result of the nutrient matrix, stimulating the growth of shoots. The authors suggest that addition of GA_3 (gibberellic aid) into the recovery medium could improve the protocol efficiency (Kulus and Abratowska 2017).

2.2 *Artemisia*

Several medicinally active components of *Artemisia vulgaris* L. have been identified, i.e., vulgrin, quercetin, coumarins, sesquiterpene lactones, volatile oils, and insulin. Since the harvest of medicinal plants from their natural habitats is leading to a depletion of plant resources, the conservation of these valuable genotypes is imperative. Therefore, research is conducted for cheap mass propagation of elite artemisia genotypes to meet the ever-increasing demand of the pharmaceutical industry (Sudarshana et al. 2013).

Low-temperature storage can greatly minimize the cost of maintaining germplasm collections because of the reduced need for manual labor due to less frequent subculturing. It also lessens the possibility of genetic variation occurrence (Kulus and Zalewska 2014a). The temperature requirement for optimum viability differs among species. In general, 4 °C is reported to be most suitable for alginate bead storage (Sharma and Shahzad 2014). Therefore, in the study by Sujatha and Kumari (2008), nodal segments (4–6 mm long) were coated with 1, 1.5, 2, 2.5, and 3% sodium alginate. The synthetic seeds were then stored at 5 ± 1 °C for 0, 20, 40, and 60 weeks. Since explant growth is not desirable during storage, therefore, this step was carried out in dark. The highest (2.5 and 3%) sodium alginate concentrations

were difficult to decant, and the latter one significantly reduced the length of roots produced. This phenomenon can be explained in two ways: (1) the actual physical barrier of the denser matrix may have delayed the regeneration of the root or (2) the higher concentrations of sodium from sodium alginate caused a change in water potential, resulting in less water for root growth. On the other hand, low concentrations (1.0 and 1.5%) of sodium alginate coated the nodal segments poorly, probably due to a reduction in its gelling after exposure to high temperature during autoclaving. Therefore, an optimum 2.0% sodium alginate was determined, as the produced beads held the nodal segments in place and still provided enough resistance to external mechanical pressure for ease of handling. All encapsulated nodal segments survived 20 weeks of 5 °C storage. Moreover, 85% of encapsulated nodal segments survived refrigerated storage for 60 weeks and, after additional 3 months on germination medium, the nodal segments produced normal shoots. In comparison, after 4 weeks of storage at 5 °C, the control (naked) nodal segments were dead and had completely dried up (Sujatha and Kumari 2008).

In another study by Sudarshana et al. (2013), encapsulated somatic embryos of *A. vulgaris* showed signs of germination after 6–7 days of culture on MS (Murashige and Skoog 1962) medium. The efficiency of the protocol was again dependent on the sodium alginate concentration. It was reported that beads formed with high concentration (4%) of sodium alginate, dropped in 75–80 mM $CaCl_2$ (calcium II chloride) for 20–30 min, were too hard for somatic embryos to germinate (53% conversion). On the other hand, 84% plantlet development rate was reported after coating the embryos with 2% sodium alginate. Moreover, embryo conversion frequency depends on storage period and temperature. Higher germination of encapsulated explants was recorded at the shortest (1 month) storage period, i.e., 64.3, 71.0, and 88.7% conversion at 4 °C, 20 °C, and 25 °C, respectively. With further increase in storage period, a decline in germination percentage was reported. This decrease may be due to oxygen deficiency and drying of gel capsule (Sudarshana et al. 2013).

2.3 *Atractylodes*

Atractylodes macrocephala Koidz. is a commonly used Chinese medicinal plant. It has long been cultivated without variety breeding, which results in the gender degradation, the decline in the output and quality of the plants, and the instability of its chemical components (Wang et al. 2011). Tissue culture technique can be used in rapid propagation of *A. macrocephala*, but high costs and inconvenient storage and transport are serious limitants of this approach (Kulus 2015a). Synthetic seed technique allows to overcome those problems.

In the study by Wang et al. (2011), nodal segments and axillary buds were encapsulated in sodium alginate (2–3.5%) with an addition of various PGRs (plant growth regulators) and nutrients into the synseed endosperm. The axillary buds were superior to stem fragments because they had fully developed “growing points” that were easily activated in the presence of sufficient external nutrients. It should be

noticed though that endogenous phytohormones of *A. macrocephala* buds were inadequate to stimulate the growth of synthetic seeds, and therefore some nutrients must be added into the gel matrix. The cytokinin BA (benzyl adenine) promoted the seeds germination at early stage, but it inhibited root regeneration. On the other hand, IAA (indole-3-acetic acid) auxin promoted both the seed's germination and rooting. Also, the addition of carbon source, e.g., sucrose or glucose, or ½ (half-strength) MS nutrients into the artificial endosperm affected positively the seed's germination and rooting steps (82% conversion), which were additionally significantly shortened as compared to water-based gelling matrix.

2.4 *Chrysanthemum*

Chrysanthemum × *grandiflorum*/Ramat./Kitam. (syn. *C. morifolium* Ramat.) is the second most popular ornamental plant species on the horticultural market. Due to self-incompatibility and (partial) sterility, the species does not produce viable seeds or fertile pollen; therefore, in vitro cultures are commonly applied with this plant for the purpose of mass reproduction and breeding (Teixeira da Silva and Kulus 2014). The synthetic seed technology was first developed with chrysanthemum in 2005 (Pinker and Abdel-Rahman 2005). It is also widely utilized for cryopreservation of the species in the encapsulation-dehydration and encapsulation-vitrification techniques (Jeon et al. 2015; Kulus 2015b).

Since the species is susceptible to mutation occurrence (there are several hot spots identified in the chrysanthemum genome); therefore, the use of meristematic explants is more preferred to produce true-to-type plants (Zalewska et al. 2011). Kulus and Zalewska (2014b) investigated the influence on various germination media on the morphogenetic potential of encapsulated shoot tips of four chrysanthemum cultivars of the Lady group. The highest germination (89%) of the encapsulated shoot tips after 90 days of storage in culture was reported on cytokinin (0.44 μM BA or 0.46 μM KIN)-supplemented medium, with no differences in regard to cultivar or culture duration. There was no influence of the medium composition on the morphometric parameters of the plants (i.e., shoot and root length or weight) in most cases. However, the presence of BA stimulated the proliferation of multiple shoots from a single explant. On the other hand, KIN (kinetin) was more effective in stimulating spontaneous rooting of shoots. Nonetheless, it was reported that encapsulation in general had a negative impact on rooting and an additional transfer of the recovered shoots on the IAA-supplemented (11.42 μM) medium was necessary to obtain 75–90% rhizogenesis efficiency.

Usually artificial seeds are produced in sterile conditions, in a laminar airflow chamber, which requires considerable costs associated with disinfection, tissue culture experts, purchase of expensive facilities, equipment and consumables, and further acclimatization of micropropagated plantlets (Kulus 2015a). All of this restricts the practical application of the encapsulation technology. The utilization

of non-aseptic conditions, i.e., ex vitro direct sowing, can greatly increase the potential for large-scale production of synthetic seeds.

For example, Pinker and Abdel-Rahman (2005) achieved 45% plantlet development (i.e., formation of shoots with roots) from double-layered synseeds on non-sterile vermiculite substrate when the second layer of calcium alginate contained only water. Numerous contaminations were reported when mannitol and/or MS medium were incorporated into the second layer. This is because despite MS macro- and microelements contain inorganic compounds, however, vitamins and sugar added together with MS salts are organic substances which can stimulate microbial contamination development.

Also Hung and Dung (2015) made an attempt of preparing and sowing synseeds of chrysanthemum in non-aseptic conditions. They embedded in vivo-grown shoot tips in 2.5% calcium alginate which were then sowed on vermiculite substrate and maintained in a polyethylene propagation chamber. It was reported that the presence of MS vitamins and 3% sucrose, either in the artificial endosperm alone or in both endosperm and vermiculite, caused bacterial and fungal contamination (already in the first week of sowing) in all synseeds and complete plant growth inhibition. On the other hand, in the absence of those organic compounds in both the endosperm and commercial substrate, chrysanthemum explants started to sprout into single shoots in the first week after sowing, and rapid rooting was detected within 2–4 weeks. Complete plantlets (70% conversion rate) were produced after 5 weeks. Moreover, removal of organic compounds from the gelling matrix and substrate stimulated the development of plantlets with more leaves, longer shoots and roots, and greater fresh and dry biomass. The presence of MS vitamins and 3% sucrose in vermiculite only resulted in a reduced contamination level (49%) and also lower plantlet formation frequency (34%). The findings by Pinker and Abdel-Rahman (2005) and Hung and Dung (2015) suggest that elimination of organic compounds is essential for wide-scale in vivo application of artificial seeds witch chrysanthemum.

Physical barrier provided by the gel matrix can also be used in the storage of explants at cryogenic temperatures, c.a. −196 to −135 °C. Zalewska and Kulus (2014) and Kulus (2018) reported that chrysanthemum shoot tips can be precultured for 7 days on the MS medium supplemented with 10 μM ABA (abscisic acid; which increases the explant stress resistance), encapsulated in 3% calcium alginate, and, after further osmotic and physical dehydration (to a level of approximately 30–40% initial moisture content), stored in liquid nitrogen. Water removal is necessary to avoid lethal ice (re)crystallization during cooling and rewarming. As for the recovery, cryopreserved material should be transferred to a water bath at 38 ± 1 °C for 3 min and, next, cultured on the MS medium supplemented with 1.16 μM KIN (Zalewska and Kulus 2013). By those means, indefinitely long-term storage of biological material is possible, without cell damage or morphology alternations (Kulus et al. 2018).

2.5 *Cineraria*

Cineraria maritima L., syn. dusty miller or silver dust, is an important annual exotic medicinal herb and ornamental species with the need of conservation. Typically, lower temperatures, e.g. refrigeration, (deep)freezing, or cryopreservation, are recommended for the purpose of biological material storage. However, Srivstava et al. (2009) developed a protocol for medium-term storage at room temperature of encapsulated microshoots (shoot tips and nodal segments) of the species. After 6 months of storage at 25 ± 2 °C, the encapsulated *C. maritima* explants were capable of recovery within 2 weeks of subsequent culture. The only requirement was moisturizing the environment by spraying the stored synseeds with sterile distilled water.

The multiplication rate (number of new shoots produced from a single donor plant) is one of the most important factors determining the micropropagation efficiency. Among the encapsulated *C. maritima* explants, 33 and 28% revealed multiple and single shoot formation per encapsulated microshoot, respectively (Srivstava et al. 2009). The recovery of a single or multiple shoots from one encapsulated explant results from the presence of one or more primordia on the explant, as observed with chrysanthemum (Kulus et al. 2018). Unfortunately, the authors did not provide the exact number of multiple shoots produced.

Usually, plantlets produced in the micropropagation process are of high quality due to lack of natural pathogens, presence of all required nutrients, etc. However, shoot hyperhydration or callusing is among the major concerns of in vitro-produced plant material. In the study by Srivstava et al. (2009), only 9% of growing shoots showed some form of abnormal phenotype, e.g., hyperhydration.

Another issue is the often reported problem with spontaneous rhizogenesis and the necessity of including an additional rooting step to the micropropagation protocol (Kulus and Zalewska 2014a). Also in the study with *C. maritima*, only 11% of developing shoots exhibited simultaneous rhizogenesis. As for the non-rooted shoots, root development was induced after 2–3 weeks of culture on root inducing medium, i.e., MS with 5.37 μM NAA (1-naphthaleneacetic acid) (Srivstava et al. 2009). Auxins, such as NAA, IAA, or IBA (indole-3-butyric acid), are typically engaged in the rooting process.

Unlike natural seeds, artificial seeds should guarantee the stability and uniformity of the plants. The genetic stability of the recovered from synseeds microshoots of *C. maritima* was assessed using RAPD (Randomly Amplified Polymorphic DNA) markers. Among the 14 amplified primers, 7 generated polymorphic patterns (20.3% of scorable bands were polymorphic). Still, clustering analysis of the RAPD profiles revealed a mean similarity coefficient of 0.944. Therefore, the genetic stability of plants produced from encapsulated microshoots following 6 months of storage was confirmed (Srivstava et al. 2009).

2.6 *Eclipta*

Eclipta alba (L.) Hassk is a popular herb of tropical, subtropical, and temperate climate. It is currently overexploited from the wild to meet up the pharmaceutical demand as a treatment for liver diseases and skin and memory disorders. Moreover, it naturally grows in wet and moist habits, which makes it vulnerable to diseases and pests. For those reasons, it is necessary to develop alternative reproduction methods of the species to avoid using contaminated pharmaceutical raw material (Singh et al. 2010). Synthetic seeds can be used to exchange germplasm of elite genotypes and axenic plant material between laboratories and pharmaceutical companies.

In the study by Ray and Bhattacharya (2010), shoot tips and nodal segments were encapsulated in 3% calcium alginate. After storage and recovery on the MS medium, the explants formed first shoots and then roots. Therefore, one-step conversion into full plantlets was achieved without the need of adding PGRs. Both explant types demonstrated similar utility for encapsulation. The artificial seeds incubated at 12 °C and 20 °C could not be stored past 4 weeks, due to the rapid revival of metabolic activities in encapsulated explants. Storage at 4 °C, on the other hand, was possible for even 8 weeks with 82.6–85.4% conversion frequency. Reduction of the sucrose concentration in the artificial matrix from 3 to 1–2% extended the storage period up to 15 weeks (40–44% conversion rate), but lower carbohydrate concentrations had a deleterious effect. The recovered plantlets were morphologically similar with the donor plants. This uniformity was confirmed by nine RAPD amplified primers (Ray and Bhattacharya 2010).

In another study, nodal segments from in vivo-grown plants of *E. alba* were used to initiate in vitro cultures. The explants were disinfected with 70% ethanol (30 s) followed by 0.05% mercuric chloride (3–4 min) and rinsed four to five times with sterile distilled water under aseptic conditions. Next, the nodal segments were embedded in various concentrations of sodium alginate (2.0–5.0%) and calcium II chloride (20–150 mM). After polymerization, the beads were washed three to four times with sterile distilled water. This is a typical step required to remove the excess of $CaCl_2$ and stop further hardening of the bead. The beads were stored in cold room on various media, i.e., PGR-free MS containing 0.88 μM BA or 0.92 μM KIN. A gelling matrix of 3.0% sodium alginate and 100 mM $CaCl_2$ was found most suitable for isodiametric bead formation. The use of double distilled water for preparing artificial endosperm reduced the shoot development as compared to liquid MS medium (devoid of calcium). Conversion into complete plantlets occurred after 4 weeks of culture, irrespectively of medium composition (93.2–100%). Moreover, encapsulated explants exhibited higher conversion rate in comparison to non-encapsulated ones (90.1–93.8%). The recovered plantlets with six to eight fully expanded leaves (3–5 cm in length) were transferred to plastic pots with soil and covered with a polyethylene bag to maintain high humidity and irrigated with tap water (Singh et al. 2010). Additional cover with mesh is also often applied to protect plants from the sunlight, as microshoots often have dysfunctional stomata

and reduced cuticle thickness. Acclimatization efficiency of plantlets reached 90% and no morphological variation was reported (Singh et al. 2010).

2.7 *Gerbera*

Gerbera daisy (or Barberton Daisy) is aimed at producing plants at a very high multiplication rate, as the species is in high demand worldwide. In the study by Taha et al. (2009), microshoots of *G. jamesonii* Bolus ex Hooker were produced from petiole explants cultured on MS medium with 13.32 μM BA, and the indirect somatic embryos were induced from leaf explants cultured on MS medium fortified with 4.44 μM BA and 0.54 μm NAA with the addition of 50 mM L-proline. Next, the explants were coated with sodium alginate (2.0–6.0%) and hardened in $CaCl_2$ (25–125 mM). It was reported that the most optimal sodium alginate concentration was 3 and 4%, and the best $CaCl_2$ solution concentration was between 100 and 125 mM.

Encapsulated microshoots responded somewhat better to encapsulation than somatic embryos. It was necessary, however, to fortify the artificial endosperm with not only MS medium salts (calcium-free) and 3% sucrose but also with 8.88 μM BA and 2.69 μM NAA. Otherwise, the germination and survival rates of the synthetic seeds were lower as compared with the non-coated control. The encapsulated microshoots and somatic embryos could have been stored at 4 °C for 60 days without viability loss (90 and 70%, respectively). Three-month storage also demonstrated good germination at 77% (encapsulated microshoots) and 60% (encapsulated somatic embryos). Longer storage, however, leads to a continuous decline in the synseed germination rate to 13 and 7% after 180 days. This is probably due to inhibited respiration of plant tissue by alginate. No phenotypic changes of the recovered plantlets were reported (Taha et al. 2009).

2.8 *Glossocardia*

The need for medicinal and aromatic plants of the *Glossocardia* genus has increased in the recent years because of the renaissance of natural, plant-dependent pharmaceuticals and cosmetic industries, which harvest plant mostly from their natural habitat (Alatar et al. 2017). Therefore, in vitro multiplication and ex situ conservation, supported also by the artificial seed technology, are important.

Composition of the gel matrix has an essential impact on the synseeds' germination capacity and should be carefully optimized. Ion exchange between Na^+ and Ca^{2+} determines its bonding, as well as the degree of rigidity of synthetic seeds (Kundu et al. 2018). A too low concentration of sodium alginate may prolong bead forming time; not provide enough protection of the explant, as beads are without a definite shape and are too soft to handle; or lead to tissue hyperhydration. On the other hand,

too high concentration may delay or even suppress the growth of plant material and limit the uptake of nutrients. Therefore, alginate concentration (1–4%) in 50–100 mM $CaCl_2$ range needs to be optimized depending on the explant (Rihan et al. 2017).

Geetha and Gopal (2009) investigated the influence of various sodium alginate concentrations (1.5–3.5%) and recovery media on the germination of synthetic seeds from embryoids of *Glossocardia bosvallea* (L.f.) DC—a medicinal annual herb. The best result (67% germination) was obtained in 2.5% sodium alginate, i.e., a concentration slightly lower than traditionally used (3%). On the other hand, germination was nil in the synthetic seed encapsulated with 1.5 and 3.5% sodium alginate. As for the recovery medium, the frequency of artificial seeds germination in MS medium was significantly higher than that of seeds cultured on Nitsch and Nitsch (1969) medium. Germinated synseeds with well-developed shoot and roots were successfully acclimatized to greenhouse conditions.

2.9 *Hypochaeris*

Hypochaeris radicata L. (hairy cat's ear) is an edible, perennial herb native to South Africa. Due to the presence of many bioactive compounds of medicinal importance, the species is used in the treatment of jaundice, rheumatism, dyspepsia, constipation, hypoglycemia, and kidney-related problems (Jamuna et al. 2014). Unfortunately, the species seed longevity is poor under natural conditions, hence affecting negatively the population sizes. The production of synthetic seeds and sowing them during appropriate period is a possible solution of this problem. Preparation of synseeds, however, requires proper explant selection.

Senguttuvan and Subramaniam (2014) produced artificial seeds of *H. radicata* from in vitro-derived leaf, root, and callus (leaf-derived) explants. Among the tested sodium alginate concentrations (1–6%); 3% was optimal. The highest shoot regeneration (over 86%) was reported with encapsulated leaf segments stored at 25 °C in the MS medium supplemented with 8.88 μM BA (which had a superior impact on shooting in comparison to other PGRs). Over 88% of explants produce multiple shots, with a mean 15.6 shoots per explant. This may indicate that BA is an important growth regulator for caulogenesis initiation, as observed by other researchers (Kulus and Zalewska 2014b; Kulus and Abratowska 2017). Interestingly, explant shooting frequency was lower if stored at 4 °C, regardless of the storage period and explant type. Rooting of shoots was well performed (73.5% efficiency) on MS medium with 5.37 μM NAA, which was more effective as compared to IAA and IBA in terms of rooting rate, number of roots per shoot, and root length. The in vitro-regenerated plantlets were acclimatized successfully (70% survivability) to glasshouse conditions using garden soil, sand, and vermicompost (1:1:1 v/v) (Senguttuvan and Subramaniam 2014).

2.10 *Sphagneticola*

Sphagneticola calendulacea (L.) Pruski is a perennial herb. The unsustainable use of this medicinal species from the wild has led to its habitat threat. Moreover, the low seed viability and seed-borne diseases make traditional propagation inefficient. The synthetic seed technology, on the other hand, is a dependable system enabling transportation and storage of explants and, thus, satisfying the needs of the pharmaceutical industry (Kundu et al. 2018).

In the study by Kundu et al. (2018), various sodium alginate (2–4%) and calcium II chloride (75 and 100 mM) concentrations were evaluated. The ideal beads were produced through a combination of 2.5% sodium alginate polymerized with 75 mM $CaCl_2$. As for the recovery medium, the full-strength MS had been reported to be optimum by many researchers (Geetha and Gopal 2009; Kamińska et al. 2018). However, with *S. calendulacea* the highest regeneration rate was achieved on ½ MS medium. Furthermore, liquid ½ MS medium was superior in respect to regrowth rate (88.8%) than semisolid ½ MS (86.6%). This is probably a result of better absorption of nutrients from the liquid state.

One of the biggest problems of encapsulated explants is the conversion into full plantlets, i.e., simultaneous caulogenesis and rhizogenesis. Media without PGRs, regardless of state and strength, were unable to stimulate shooting and rooting of the explants at the same time. On the other hand, ½ MS with the addition of various concentrations of BA at 0.44–8.88 μM and NAA at 1.34–5.37 μM allowed for one-step formation of complete plantlets. Optimal combination (4.44 μM BA and 1.34 μM NAA) provided 91.1% regeneration, as well as 6.6 shoots and 3.1 roots per encapsulated nodal segment. It is also beneficial to add sucrose (1.5–3.0%) into the bead matrix and/or germination medium. Sucrose is a carbohydrate and energy source for plants in tissue culture, and its deficiency in the medium may completely inhibit synseed germination. On the other hand, too high carbohydrate concentration (above 2.5%) may also temporarily suppress explant development which can be used in short-term conservation of the species desirable genotypes (Kundu et al. 2018).

In the study with *S. calendulacea*, it was reported that the regeneration rate and pace (days taken to bead break, 8–14) of artificial seeds decreased slowly with a prolongation of storage duration, both at 8 and 25 °C. However, this decrease was much more drastic at the latter temperature (88, 26.7, and 0% regeneration rates after 30, 60, and 90 days, respectively). Moreover, those conditions exhibited a greater decrease in the mean shoots and root number produced by a single explant. In comparison, over half of the synseeds stored at 8 °C for 90 days were able to germinate, confirming the beneficial influence of refrigeration (Kundu et al. 2018).

Sowing of artificial seeds directly in non-sterile condition is a much more cost-effective practice. The best regeneration frequency (42.2%) of *S. calendulacea* synthetic seeds, under ex vitro conditions, was recorded on sand, soil, and vermicompost (1:1:1, w/w) irrigated with ½ MS salts without sucrose. The pretreatment of excised node segments on agar-solidified ½ MS medium with

19.6 μM indole-3-acetic acid (IBA) in dark for 24 h prior to encapsulation facilitated the explants to form root meristems after sowing.

Evaluating the genetic homogeneity of the recovered plantlets is essential to conclude the success of a procedure. RAPD and ISSR (inter-simple sequence repeats) are among the most popular molecular markers due to their simplicity, relatively low cost, and whole-genome screening ability. In the study by Kundu et al. (2018), the genetic uniformity of the regenerants from synthetic seeds after 90 days of storage at 8 and 25 °C was confirmed by those two marker types.

2.11 Spilanthes

Most of the available literature is focused on the synthetic seed production of the *Spilanthes* genus: *S. acmella* (L.) Murr. and *S. mauritiana* DC.

Spilanthes acmella is a threatened medicinal and pesticidal species, documented for its antibacterial, antifungal, and antimalarial activity, grown in tropics and subtropics. Due to the presence of numerous valuable secondary metabolites, the species is of great demand on the cosmetic and pharmaceutical markets. Hence the need for large-scale propagation of the species. Unfortunately, traditional reproduction through seeds is limited by their low viability, seasonal dependency, low germination rates, and high level of heterozygosity (Devi et al. 2012). Therefore, developing efficient micropropagation protocols is desired.

Singh et al. (2009) were the first to describe an encapsulation technology of *S. acmella* shoot tips (3–5 m in length) excised from in vitro-proliferated shoots. The encapsulated in 3% calcium alginate explants could be stored at 4 °C up to 60 days. It was found that the presence of full-strength MS salts in the gel matrix was superior over the double distilled water for sprouting, although half-strength MS medium was found best for root growth. Interestingly, the addition of 2.2 μM BA to the artificial endosperm caused significantly worse plantlet conversion. Shoots and roots broke the capsule within 1 week; however, conversion into complete plantlets took 4–5 weeks of culture in liquid MS medium. Culture on solid planting media was less effective. This is beneficial since liquid medium is preferable for micropropagation as it reduces expenditures; agar is one of the most expensive media components. An often observed problem is the decline of synseed viability with increasing storage duration at refrigeration (4 °C), which was also reported by Singh et al. (2009). The plantlet conversion ability decreased from 97 to 100% after 15-day storage to 50% after 60-day storage. Still, plantlets regenerated from synseeds were successfully acclimatized to ex vitro conditions (90% survival) in a mixture of sand and garden soil (1:1) during 30 days.

Even though the described protocol was successful, not many shoot tips can be excised from a single mother plant. Therefore, utilization of nodal segments with axillary buds can be more effective, especially with species which do not have a developed somatic embryo induction protocol.

Sharma et al. (2009a) found that among the tested sodium alginate and calcium II chloride concentrations (1–5% and 25–200 mM, respectively), 4% and 100 mM were optimal for the encapsulation of *S. acmella* single-node explants in isodiametric beads. As for the PGRs added to the germination medium (BA with IAA, IBA, or NAA), the combination of BA and NAA proved to be most efficient; regrowth was observed after 2–3 weeks of culture. It was possible to store the encapsulated node segments at 4 °C for even 8 weeks (46% conversion) in parafilm-sealed sterilized beakers, provided that the encapsulation matrix was enriched with MS salts. Another possibility was direct sowing of synthetic seeds to ex vitro conditions on Soilrite™ substrate moistened with ¼ (quarter-strength) MS salts (63% conversion rate). This approach could be useful in developing a cost-effective propagation system for *S. acmella*.

In the study by Devi et al. (2012), it was found that the presence of SH salts (Schenk and Hildebrandt 2002) with 4.44 μM BA in the artificial endosperm stimulated the formation of longer shoots and roots in comparison to MS medium-based beads. On the other hand, when IAA (2.9 μM) was incorporated with cytokinin in the encapsulation matrix, the overall frequency, number, and length of shoots and roots declined, irrespective of the basal media used. Addition of 2.9 IAA and 2.22 μM BA to the germination MS medium, however, was superior over KIN and BA in higher concentrations in reducing the time needed for multiple shoot formation (6 days) from encapsulated nodal segments (25 shoots from a single explant). Those differences in PGRs activity may be explained by their different translocation rates and metabolic process in which the PGRs can be degraded or conjugated with other compounds (Singh and Chaturvedi 2010).

Spilanthes mauritiana is an endangered herb with antimicrobial activity, native of Eastern Africa. Traditional vegetative reproduction of the plant via cuttings is inefficient; therefore, in vitro tissue cultures should be employed with this species (Sharma and Shahzad 2014).

Sharma et al. (2009b) investigated the influence of germination medium strength and storage period on the conversion (shoot and root formation) of encapsulated *S. mauritiana* axillary buds. The synseeds remained high viability (over 80%) for up to 4 weeks of storing at 4 °C, which was the longest investigated storage period. Therefore, longer storage is probably also possible. Interestingly, the highest conversion rate of synseeds was reported on the half-strength MS medium; both full-strength and quarter-strength MS were less effective.

Sharma and Shahzad (2014) focused on the development of a synthetic seed protocol for the purpose of short-term storage and germplasm exchange of *S. mauritiana* nodal segments. Under in vitro conditions, sprouting started within 8–10 days of incubation on MS basal medium. The simultaneous development of shoot and root was observed, which resulted in rapid growth of plantlets after 5–6 weeks. It should be noticed, however, that the induced roots were very thin and few in number which resulted in poor survival during acclimatization in Soilrite™. Therefore, addition of auxin and cytokinin into the germination medium was necessary. Interestingly, regardless of the PGR composition, only single shoots with roots were produced. Among the combinations evaluated, MS medium with BA

(1.0 μM) and IAA (0.5 μM) guaranteed maximum conversion, greatest shoot length, maximum root number per synseed, and greatest root length, without any intervening callus. The acclimatization efficiency of such produced plantlets reached 90%.

Sharma and Shahzad (2014) also assessed the possibility of ex vitro synseeds sowing and found that among various planting substrates, Soilrite™ moistened with ¼ MS salts was most optimal with 63.4% conversion frequency. In such conditions, shoot sprouting was noticed after 2 weeks of sowing followed by rooting after 3 weeks. Soilrite™ moistened with tap water was less effective, probably because of reduced nutrient availability. Similarly, soil was not as effective because of its compact texture as compared to the Soilrite™.

2.12 Stevia

Stevia rebaudiana Bertoni is one of the most well-described species in terms of encapsulation and synthetic seeds production. Its cultivation is popular worldwide as the only zero-calorie sugar substitute which is 300 times sweeter than cane sugar. Unfortunately, stevia seeds show a very low germination rate, whereas vegetative propagation is limited by lower number of individuals produced and long time needed. Due to the intensive exploitation and low propagation response, this medicinal plant became endangered (Nower 2014).

Andlib et al. (2011) focused on the importance of artificial matrix composition when preparing *S. rebaudiana* synseeds. They found that preparation of beads based on MS medium salts is more efficient (70% plantlet development) than on distilled water (55% conversion). MS medium was also more effective for germination than White's medium.

Besides sodium alginate and calcium chloride concentrations, also the treatment time has a significant influence on the quality of the obtained beads and, thus, of the whole conservation or propagation procedure. In the study by Ali et al. (2012), shoot tips and nodal explants were placed for different time durations in sodium alginate and calcium II chloride. It was found that the duration of sodium alginate treatment has no effect on bead formation. On the other hand, timing of $CaCl_2$ treatment proved to be crucial. With 100 mM $CaCl_2$, 15 min is suitable for isodiametric bead formation, whereas shorter treatment led to fragile bead formation, while after longer periods, the beads became very hard and the explants dried and died. Ali et al. (2012) also investigated the effect of chemical pretreatment on synthetic seed germination. They subjected the synseeds to self-breaking treatment by dipping them in 200 mM potassium nitrate solution for 5 min. This procedure leads to the swelling of seeds and faster sprouting (within 1 week) in comparison to non-pretreated ones (sprouting after 30 days). The highest conversion rate after 1 week (100%) was reported in the liquid MS medium supplemented with 4.44 μM BA. Solid media were less effective. Root regeneration was achieved after 15 days without the addition of any auxins. Frequency to plantlet conversion of artificial seeds decreased gradually as the storage duration at 4 °C increased (from 63.3% after 15 days to 10% after

60 days). Also the time required for germination increased from 6 days (after 15-day storage) to 16.6 days (after 60-day storage). This suggests explant damage during medium-term storage.

Khan et al. (2013) found that ideal isodiametric, compact, clear, and uniform size of beads with nodal segments can be produced with 4% sodium alginate and 75 mM calcium II chloride. Germination of *S. rebaudiana* synthetic seeds occurred within 8 days after inoculation on solid MS medium (60% germination), followed by moist cotton (25–40%) and soil mixture (10–30%). Interestingly, addition of BA, NAA, and adenine sulfate to the germination media worsened the results (10–20% germination). In vitro multiplication of shoots produced from synseeds was reported when subcultured on MS medium with 9.29 μM KIN and 40 mg L^{-1} adenine sulfate. Since shoot tips and axillary buds are unipolar structures with no root meristem, therefore, transfer on the rooting medium was necessary. Among the tested NAA and IBA auxin concentrations (2.46–8.06 μM), the best rooting with the maximum length of roots was reported with 5.37 μM NAA.

In order to produce a suitable number of *S. rebaudiana* shoot tips, Nower (2014) induced multiple shoots formation (22.75 shoots per one explant) from nodal segments inoculated in MS medium supplemented with 4.44 μM BA and 2% fructose. The shoot tips were encapsulated in 4% sodium alginate (based on MS liquid medium) and 100 mM $Ca(NO_3)_2 \cdot 4H_2O$ (calcium nitrate tetrahydrate). Further culture in a germination medium of various strength (¼, ½, ¾, and full MS) supplemented with 1.11 μM BA was investigated. Moreover, the study focused on inducing osmotic stress (by adding mannitol or sorbitol to the germination medium) in order to achieve reduced growth. The best results (100% conversion rate) were achieved after storing the explants for 5 weeks on full-strength MS medium as compared with other treatments. The addition of 0.05 M mannitol to the medium increased culture survival but with deterioration of shoots' quality and growth. Higher concentrations of osmotic agents had a negative impact on the culture survival. Transfer of germinated shoots on the auxin-supplemented medium stopped shoot multiplication and supported nearly 100% rooting. Application of MS medium fortified with 1.14 μM IAA resulted in the maximum number of roots formed per one explant, while 4.92 μM IBA resulted in longest root regeneration. Longer roots, however, may be more "problematic" during acclimatization.

Maintaining the biochemical stability is an important aspect during plant material storage. Lata et al. (2014) investigated the effect of encapsulation on the stability in the chemical profile and quantification of rebaudioside A and stevioside content and found no differences between the mother and regrown plants following in vitro storage, confirming the dependency of the synseeds technology.

2.13 Taraxacum

Light can be a deleterious factor during storage. Therefore, Kamińska et al. (2018) focused on the influence of light conditions on the conversion ability and *post-*

storage regrowth of the synthetic seeds of *Taraxacum pieninicum* Pawł.—a critically endangered Polish species.

It was reported that synseeds produced from shoot tips can be stored at 4 °C even for 12 months without subculture. In comparison, traditional in vitro cultures need to be subcultured every 4 weeks, which is associated with the risk of contamination and additional labor costs. Time of storage did not affect the survival of synseeds, nor the quality of the produced plantlets. The cultures, however, should be kept in dark, as light, even at reduced intensity, is a stress factor causing numerous necroses and decreased shoot ability to proliferate in the first subculture, probably due to the increase in the ABA/GA_3 ratio. A mean of 12.2 shoots from a single explant was produced after 12-month storage on the proliferation medium containing 1.1 μM BA and 0.14 μM NAA (Kamińska et al. 2018).

In the study by Kamińska et al. (2018), no ploidy level change was reported (flow cytometry analyses), regardless of storage conditions. This suggests that slowing down and speeding up the metabolism of *T. pieninicum* during cold storage and regrowth, respectively, do not interfere with cell division. Moreover, all regenerants from encapsulated and stored shoot tips were genetically similar to the non-cultured control plants, as confirmed by 21 RAPD primers. It can be assumed that the synseed technology minimizes the risk of somaclonal variation occurrence, which is a major problem in traditional tissue culture (Miler and Zalewska 2014). One should keep in mind that even though the growth of the explants in cold conditions is reduced, still prolonged exposure to low temperature may cause stress or variation, especially to thermophilic species, due to the generation of reactive oxygen species (ROS) (Kamińska et al. 2018).

The described synthetic seed production protocol is very effective, as it allows to overcome the problem of physiological dormancy of natural seeds, which normally require stratification for germination.

3 Conclusions

In vitro tissue culture is a dependable biotechnological tool for the rapid reproduction of Asteraceae plants for the purpose of micropropagation, preservation, and breeding, especially for species with limited reproductive capacity.

Synthetic seeds possess the ability to convert into a plant under in vitro or in vivo conditions and also retain this potential after storage. They have numerous applications, including multiplication of non-seed-producing plants, polyploids or ornamental hybrids, propagation of male or female sterile plants, and germplasm preservation.

Despite the great progress done over the past 30 years, the synthetic seed technology still has some issues which need to be considered, such as synthetic testa and endosperm composition standardization, too low conversion rate in ex vitro substrate or after longer storage, and the fact that most of works are operated manually. It is also worth to consider other gelling agents in future research, e.g.,

carrageenan, gellan gum, sodium pectate, poly(ethylene glycol), poly(vinyl alcohol), and carboxyl methyl cellulose. Nevertheless, the synthetic seed technology can be successfully applied with the Asteraceae plant species.

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Synthetic Seeds: A Valuable Adjunct for Conservation of Medicinal Plants

Neelam Sharma, R. Gowthami, and Ruchira Pandey

Abstract Encapsulation technology is an upcoming powerful tool for plant propagation and germplasm conservation. It is expected to impact exchange of plant materials among laboratories within and across countries in a more economical and convenient way with lesser quarantine restrictions.

For synthetic seed production, alginate-encapsulated in vitro-derived shoot tips and nodal segments are preferred propagules over somatic embryos. It is an efficient technique for clonal propagation, germplasm conservation, and exchange of valuable plant genetic resources. Concerted efforts are required to apply above technique to strengthen the ongoing efforts towards conservation of medicinal and RET plants.

In some plants encapsulated shoot tips/nodal segments conserved in a cryovial without any medium offer tremendous scope especially for exchange and need to be applied to other plants. This technique once developed can contribute significantly to the conservation of germplasm diversity, as it requires minimum inputs and infrastructure. Additionally encapsulated propagules facilitate cryopreservation using encapsulation-dehydration and/or encapsulation-vitrification technique. Though an important aspect of any in vitro program, there is limited information on the genetic stability assessment of synthetic seed-derived (before and after conservation) plantlets.

Keywords Synthetic seeds · Medicinal plant · Conservation · Germplasm exchange · Cryopreservation · Encapsulation-dehydration · Encapsulation-vitrification

N. Sharma (✉) · R. Gowthami · R. Pandey
Tissue Culture and Cryopreservation Unit, ICAR-National Bureau of Plant Genetic Resources (NBPGR), New Delhi, Delhi, India
e-mail: neelam.sharma1@icar.gov.in

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1 Introduction

Synthetic seed refers to an encapsulated propagule capable of producing a plant under suitable growing conditions. The concept of artificial seed, for "an encapsulated single somatic embryo" allowing somatic embryo to germinate so as to be used as an "artificial seed," was first proposed by Murashige (1977). However successful development of synthetic seeds was reported in early 1980s in carrot (Kitto and Janick 1982) and alfalfa (Redenbaugh et al. 1984). Realizing the distinct advantages, a number of publications dealing with various aspects of synthetic seeds on different plant species including medicinal plants have appeared in literature (Gantait et al. 2015; Sharma et al. 2013).

As many plant species fail to produce somatic embryos and owing to low success coupled with high cost of somatic embryo production in many species, Bapat et al. (1987) proposed use of in vitro-derived propagules for synseed formation, especially in non-embryo-producing species. Thus, an artificial seed (also known as synseed, synthetic seed, or manufactured seed) as per revised concept refers to an artificially encapsulated somatic embryo, shoot bud, or any other propagule that possesses the ability to form a plantlet under in vitro or in vivo conditions (Ara et al. 2000; Sharma et al. 2013). The synthetic seed technology holds great promise especially in difficult-to-conserve plant species/crops owing to lack of seeds formation or production of small quantities of seeds or production of recalcitrant seeds and/or due to their being rare, threatened, and endangered (RET) species.

The advantages of aforementioned technique include reduction in overall expenditure owing to economy of space, culture medium, and time as each bead contains a single plantlet in a small amount of nutrient medium in the encapsulating matrix. According to an estimate, a 250 ml flask can accommodate five times more encapsulated propagules than it can hold shoots (Ahuja et al. 1989). It also facilitates transfer out especially in species posing problems in rooting and ex vitro transfer of plantlets. In recent past, with the feasibility of encapsulating in vivo-derived propagules such as dormant buds, technique is being increasingly applied as tool for long-term (cryopreservation) conservation. Besides tremendous potential in the storage and conservation of propagules, it allows easy transport, transplantation, and exchange of propagules. Due to less stringent quarantine requirements, the synseed technology can facilitate germplasm exchange (Sharma et al. 2013). In addition, it is also viewed as a promising option for the management of transgenic (seedless) plants and polyploidy plants with unique traits (Gangopadhyay et al. 2011).

Medicinal plants have special significance in healthcare due to presence of secondary metabolites with high curative value. A large number of plants employed for medicinal purposes—in allopathy, traditional medicine systems, and tribal/folk practices—are collected from naturally occurring wild populations. Additionally, many of these produce limited or recalcitrant seeds that cause problem in propagation, conservation, and utilization. Only a few species of medicinal plants are under cultivation, and for majority even the reproductive biology is unknown. Indiscriminate collection from nature coupled with limited or no successful efforts of

cultivation, endemism, reduced population size, destruction of natural habitat due to anthropogenic activities, and climate change has led to decline in a number of species and, thereby, resulted in many species becoming threatened with extinction. Additionally in many species, upon cultivation in some the active principle may not be the same or may need long period to build up level of active principle. There are only few medicinal plants which have been successfully cultivated (Pareek et al. 2005), in general some factors which discourage commercial cultivation of medicinal plants besides lack of information on their propagation behavior include low seed viability/germination and long gestation periods (Pareek et al. 2005; Sharma et al. 2013; Sharma and Pandey 2013). Additionally propagation of RET medicinal plants is also constrained due to small endosperm, low germination rate, physical/physiological dormancy, nonavailability of sufficient propagules, recalcitrance to propagation techniques, etc. This has led to increasing thrust on application of in vitro techniques for propagation, conservation, and secondary metabolite production (Chandel et al. 1996; Sharma and Pandey 2013; Sharma et al. 2007). Synthetic seed technology has further opened new avenues in the field of propagation and conservation of plant genetic resources (PGR). Despite reported since three decades, the technique for artificial seed production has not been amply developed for the medicinal plants. Application of artificial seeds as a technique, with dual benefits of clonal propagation and conservation, needs attention and research thrust in this group of precious plants, before these are lost.

In literature, extensive progress made in synthetic seed technology has been reviewed either for a particular group of crops (e.g., fruit crops) or the technique/technology vis-à-vis prospects and limitation of technology (Ara et al. 2000; Kiwoska and Thiem 2011; Rai et al. 2009; Saiprasad 2001; Sharma et al. 2013). There is no consolidated information regarding application of technology for short- and long-term conservation of plant germplasm. The present chapter provides an update on progress, challenges, and application of synthetic seed technology with special emphasis on conservation of medicinal plants.

2 Synthetic Seed Technology

There are two essential components of synthetic seed: (1) *plant propagule* (in vitro- or in vivo-derived) and (2) *matrix* (a gelling material to encapsulate plant propagules) containing nutrients, antibiotics, or other essential additives.

2.1 Plant Propagule

In general and as per original concept, synthetic seed involved encapsulation of somatic embryos. With revised concept (Bapat et al. 1987), the technology was extended to encapsulation of shoot tips, shoot buds, nodal segments, embryogenic

masses, and calli as also in vitro-induced bulb, bulblets, hairy roots, and microtubers (Reddy et al. 2012; Gantait and Sinniah 2013) in different plant species. In general, the size of propagule used for encapsulation is 3–5 mm with exception of in vivo-derived winter buds.

In medicinal plants, though the work initiated later, successful encapsulation using various propagules has been reported in a large number of species (Table 1). In *Decalepis* spp. nodal segments from aseptically raised seedlings were most suitable propagules for encapsulation (Sharma and Shahzad 2012).

Though in most encapsulation studies, in vitro-derived propagules from proliferating shoot cultures were used, there are only a few reports of successful encapsulation of in vivo-derived propagules from mature plants (Sharma et al. 2013). For example, in *Curcuma amada*, synseed was produced using microshoots with a small basal rhizome portion from in vivo-grown rhizomes (Banerjee et al. 2012).

A brief account of various propagules used in synseed formation in medicinal plants is described below.

2.1.1 Somatic Embryos

Encapsulated somatic embryos (SE) can be handled like true seeds. Despite realizing the advantages of somatic embryos over other propagules, there is limited and varying degree of success with somatic embryogenesis in medicinal plants, for example, in *Dioscorea floribunda* and *D. deltoidea* (Sharma 1995; Sharma and Chaturvedi 1989), *Holostemma ada-kodien* (Martin 2003a), etc. Additionally, embryogenic ability is genotype specific (Sharma 1995), and not much work has been done on medicinal plants. Among a few medicinal plants for which SEs have been reported, synthetic seed production has been successful in *Anethum graveolens* (Dhir et al. 2014), *Arnebia euchroma* (Manjkhola et al. 2005), *Artemisia vulgaris* (Sudarshana et al. 2013), *Hemidesmus indicus* (Cheruvathur et al. 2013a), *Rotula aquatica* (Chithra et al. 2005), and *Swertia chirayita* (Kumar and Chandra 2014) (see Table 1).

2.1.2 Shoot Tips

Among various non-embryogenic explants, shoot tips—unipolar propagule without root meristem—have proven to be the most amenable for synseeds development in a number of medicinal plants such as *Adhatoda vasica* (Anand and Bansal 2002), *Bacopa monnieri* (Sharma et al. 2016), *Ceropegia* spp. (Murthy et al. 2013), *Chonemorpha grandiflora* (Nishitha et al. 2006), *Cineraria maritime* (Srivastava et al. 2009), *Coleus forskohlii* (Swaroopa et al. 2007), *Curculigo orchioides* (Nagesh et al. 2009), *Eclipta alba* (Ray and Bhattacharya 2010), *Glycyrrhiza glabra* (Mehrotra et al. 2012), *Picrorhiza kurroa* (Mishra et al. 2011), *Mentha arvensis* (Islam and Bari 2012), *Withania somnifera* (Singh et al. 2006b), etc.

Table 1 Status of synthetic seed production in medicinal plants

S. No	Plant species	Propagule	Encapsulation matrix		Regrowth (in vitro) (%)	References
			Matrix	Concentration of $CaCl_2$		
1.	*Acorus calamus*	MR	3% SA + ½ MS	3%	100	Quraishi et al. (2017)
2.	*Adhatoda vasica*	SB	4% SA + B_5 medium + 4.65 μM Kn + 50 mg/l PG	1.1%	66.28	Anand and Bansal (2002)
3.	*Allium sativum*	Calli	1.5% SA + ½ MS +5 × 10^{-6} μM NAA + 1 × 10^{-5} μM Kn	50 mM	95	Kim and Park (2002)
4.	*Allium sativum*	Bulblets	1–4% SA + 0.2% AC + antibiotic	225 mM	–	Bekheet (2006)
5.	*Allium tuberosum*	Shoot base	3% SA + MS (calcium free)	100 mM	100	Anonymous (2004)
6.	*Althaea officinalis*	NS	3% SA	100 mM	66	Naz et al. (2018)
7.	*Anethum graveolens*	SE	3% SA	100 mM	83	Dhir et al. (2014)
8.	*Angelica glauca*	SE	3% SA	100 μM (calcium nitrate)	75	Bisht et al. (2015)
9.	*Arnebia euchroma*	SE	3% SA, 100 mM (calcium nitrate)	25 mM	60.6	Manjkhola et al. (2005)
10.	*Aristolochia tagala*	MSt	3% SA	68 mM	80	Remya et al. (2013)
11.	*Artemisia vulgaris*	SE	2% SA	75 mM	90	Sudarshana et al. (2013)
12.	*Atropa belladonna*	SB	6% SA + MS + 3.0 mg/l BA + 1.0 mg/l IAA	75 mM	100	Ahuja et al. (1989)
13.	*Bacopa monnieri*	ST	3% SA	80 mM	86.6	Muthiah et al. (2013)
14.	*Bacopa monnieri*	NS	3% SA	80 mM	60	Muthiah et al. (2013)
15.	*Bacopa monnieri*	ST, NS	3% SA + MS (calcium free)	100 mM	100	Sharma et al. (2016)
16.	*Cannabis sativa*	NS	5% SA + MS + 0.5 μM TDZ + 2.5 μM IBA + 0.3–0.5% PPM	50 mM	100	Lata et al. (2009a)
17.	*Capparis decidua*	ST, NS	3% SA + MS (calcium free)	100 mM	61–91.6	Ahlawat et al. (2018)
18.	*Cassia angustifolia*	NS	3% SA	100 mM	94	Parveen and Shahzad (2014)

(continued)

Table 1 (continued)

S. No	Plant species	Propagule	Encapsulation matrix		Regrowth (in vitro) (%)	References
			Matrix	Concentration of $CaCl_2$		
19.	*Catharanthus roseus*	SE	2.5% SA + 3% S	100 Mm	84.33	Maqsood et al. (2012)
20.	*Centaurium erythraea*	TrSB	3% SA	50 mM	86	Piatczak and Wysokińska (2013)
21.	*Centaurium erythraea*	TrSB	3% SA	50 mM	56	Piatczak and Wysokińska (2013)
22.	*Centella asiatica*	AB, NS	4% SA	75 mM	85.7	Prasad et al. (2014)
23.	*Ceropegia barnesii*	NS	3% SA + MS	–	–	Ananthan et al. (2018)
24.	*Ceropegia bulbosa*	NS	3% SA	100 mM	100	Dhir and Shekhawat (2013)
25.	*Ceropegia spiralis, C. pusilla*	ST, NS	1–5	25–100 mM	86.6-90	Murthy et al. (2013)
26.	*Cineraria maritime*	ST, NS	3% SA + MS + 1.5% S	3%	82.35	Srivastava et al. (2009)
27.	*Clitoria ternatea*	SE	4% SA + MS + 3% S + 1 mg/l BA + 0.2 mg/l NAA	100 mM	92	Kumar and Thomas (2012)
28.	*Coleus forskohlii*	ST, NS	3.5% SA + MS + 2% S	1.36 g/150 ml	75	Swaroopa et al. (2007)
29.	*Curculigo orchioides*	SB	2.5% SA + MS	100 mM	68	Nagesh et al. (2009)
30.	*Decalepis hamiltonii*	NS	4% SA + MS	100 mM	77	Sharma and Shahzad (2012)
31.	*Dendrobium nobile*	PLB	3% SA	100 mM	78.2	Mohanty et al. (2013)
32.	*Dioscorea bulbifera*	NS	3% SA + MS	2.5%	70–75	Narula et al. (2007)
33.	*Dioscorea floribunda*	SB	6% SA + MS + 5.0 mg/l IBA	75 mM	100	Ahuja et al. (1989)
34.	*Eclipta alba*	ST, NS	3% SA	1.11%	94.3	Ray and Bhattacharya (2010)
35.	*Eclipta alba*	NS	3% SA + MS	100 mM	100	Singh et al. (2010)
36.	*Gentiana lutea*	SE	3% SA	100 mM	80	Holobiuc (2012)
37.	*Gentiana pneumonanthe*	ApB, ASB	3% SA in water (with or without 2.0 μM IAA) or MS	100 mM	–	Bach et al. (2004)

38.	*Gentiana scabra*	AB	3% SA	100 mM	–	Suzuki et al. (2005)
39.	*Glochidion velutinum*	ST	3% SA	100 mM	86.4	Mallikarjuna et al. (2016)
40.	*Glycyrrhiza glabra*	ST, NS	3% SA + MS	100 mM		Mehrotra et al. (2012)
41.	*Hemidesmus indicus*	SE	3% SA	75 mM	100	Cheruvathur et al. (2013a)
42.	*Hibiscus moscheutos*	NS	2.75% SA	50 mM	80	West et al. (2006)
43.	*Hibiscus moscheutos*	NS	2.75% SA	50 μM	–	Preece and West (2006)
44.	*Hyoscyamus muticus*	AS	6% SA + MS + 0.5 mg/l BAP + 1.0 mg/l IAA	75 mM	100	Ahuja et al. (1989)
45.	*Mandevilla moricandiana*	NS	3% SA + Liquid MS (without calcium)	0.1 M	83.3	Cordeiro et al. (2014)
46.	*Mentha arvensis*	ST	0.8 gm SA/20 ml of MS + 2.0 mg/l BAP + 0.5 mg/l Kn	1.4%	65	Islam and Bari (2012)
47.	*Mentha arvensis*	NS	0.8 gm SA/20ml of MS + 2.0 mg/l BAP + 0.2 mg/l NAA	1.4%	80	Islam and Bari (2012)
48.	*Mentha arvensis*	SB	6% SA + MS + 3.0 mg/l BAP + 1.0 mg/l IAA	75 mM	100	Ahuja et al. (1989)
49.	*Nyctanthes arbor-tristis*	NS	3% SA + MS	100 mM	76.66	Jahan and Anis (2015)
50.	*Ocimum basilicum*	NS	3% SA + MS	75 mM	80	Siddique and Anis (2009)
51.	*Ocimum americanum*	AB	4% SA + MS + 100 mg/l myo-inostiol + 30 g/l + 1.1 μM BA	75 mM	97	Mandal et al. (2000)
52.	*O. basilicum*	AB	4% SA + MS + 100 mg/l myo-inostiol + 4.4 μM BA	75 mM	98	Mandal et al. (2000)
53.	*O. gratissimum*	AB	4% SA + MS + 100 mg/l myo-inostiol + 2.2 μM BA	75 mM	95	Mandal et al. (2000)
54.	*O. gratissimum*	MSt	3% SA	75 mM	98.62	Saha et al. (2014a)
55.	*O. sanctum*	AB	4% SA + MS + 100 mg/l myo-inostiol + 4.4 μM BA	75 mM	96	Mandal et al. (2000)
56.	*O. kilimandscharicum*	ST	3% SA	75 mM	79.53	Saha et al. (2014b)

(continued)

Table 1 (continued)

S. No	Plant species	Propagule	Encapsulation matrix		Regrowth (in vitro) (%)	References
			Matrix	Concentration of $CaCl_2$		
57.	*Ochradenus baccatus*	–	3% SA	100	86	Al-Qurainy et al. (2014)
58.	*Phyllanthus amarus*	ST	3% SA + MS	75 mM	90	Singh et al. (2006a)
59.	*Phyllanthus fraternus*	NS	3% SA	100 mM	92.5	Upadhyay et al. (2014)
60.	*Picrorhiza kurroa*	ST, NS	3% SA + ½ MS + 1.5% S	3%	21.43	Mishra et al. (2011)
61.	*Picrorhiza kurroa*	SB	6% SA + MS	75 mM	87	Ahuja et al. (1989)
62.	*Picrorhiza kurroa*	TrHR	–	–	73	Rawat et al. (2013)
63.	*Plantago asiatica*	ST	3–3.5% SA + MS ± 1.5–3.0% S ± 0.1 mg/l BA	–	–	Andrzejewska-Golec and Makowczyńska (2006)
64.	*Plumbago indica*	TrHR	SA	–	80–90	Gangopadhyay et al. (2011)
65.	*Pogostemon cablin*	NS	4% SA + MS + 2% S	100 mM	91.1	Swamy et al. (2009)
66.	*Pogostemon patchouli*	SB	6% SA + MS + 5.0 mg/l Kn	75 mM	98	Ahuja et al. (1989)
67.	*Rauvolfia serpentina*	ST	3% SA	100 mM	68.5–100	Ray and Bhattacharya (2008)
68.	*Rauvolfia serpentina*	NS	3% SA + WPM	100 mM	80	Faisal et al. (2012)
69.	*Rauvolfia tetraphylla*	NS	3% SA + MS	100 mM	80.6	Faisal et al. (2013)
70.	*Rauvolfia tetraphylla*	NS	3% SA + WPM	100 mM	90.3	Alatar and Faisal (2012)
71.	*Rheum emodi*	AS	6% SA + MS	75 mM	–	Ahuja et al. (1989)
72.	*Rhinacanthus nasutus*	SE	4% SA + MS	100 mM	94	Cheruvathur et al. (2013b)
73.	*Rotula aquatic*	SE	3% SA + MS/½ MS (except $CaCl_2$)/1/3 MS + 3% S	50 mM	100	Chithra et al. (2005)
74.	*Rumex vesicarius*	SE	2% SA	100 mM	85	Nandini et al. (2014)
75.	*Salvia officinalis*	ST	2 or 3% SA + MS	50 mM	70	Grzegorczyk and Wysokińska (2011)
76.	*Salvia sclarea*	SB	6% SA + MS + 0.5 mg/l IBA	75 mM	91	Ahuja et al. (1989)

77.	*Salvia splendens*	NS	4% SA	100 mM	63.6	Sharma et al. (2014)
78.	*Selinum tenuifolium*	SE	6% SA + MS	75 mM	100	Ahuja et al. (1989)
79.	*Solanum nigrum*	ST	3% SA + MS	100 mM	97.2–100	Verma et al. (2010)
80.	*Spilanthes acmella*	NS	4% SA + MS	100 mM	87.8	Sharma et al. (2009b)
81.	*Spilanthes acmella*	ST	3% SA + MS	100 mM	100	Singh et al. (2009)
82.	*Spilanthes mauritiana*	NS	4% SA + MS + 3% S	100 mM	>90	Sharma et al. (2009a)
83.	*Sterculia urens*	NS	4% SA	100 mM	95	Subhashini Devi et al. (2014)
84.	*Swertia chirayita*	SE	4% SA	100 mM	84	Kumar and Chandra (2014)
85.	*Tylophora indica*	SE	3% SA + ½ MS	50 mM	22.4	Devendra et al. (2011)
86.	*Tylophora indica*	NS	3% SA + MS	100 mM	91	Faisal and Anis (2007)
87.	*Tylophora indica*	NS	3% SA + calcium free ½ MS	75 mM	93.3	Gantait et al. (2017a)
88.	*Valeriana wallichii*	SB	6% SA + MS + 5.0 mg/l Kn + 1.0 mg/l IAA	75 mM	98	Ahuja et al. (1989); Mathur et al. (1989)
89.	*Vitex negundo*	NS	3% SA + MS	100 mM	92.6	Ahmad and Anis (2010)
90.	*Withania coagulans*	ST, NS	3% SA + MS/water	100 mM	72	Rathore and Kheni (2015)
91.	*Withania somnifera*	ST	3% SA	75 mM	87	Singh et al. (2006b)
92.	*Withania somnifera*	NS	3% SA + MS/water	100 mM	86.2	Fatima et al. (2013)
93.	*Zingiber officinale*	SB	4% SA	47 mM	81	Sharma et al. (1994)
94.	*Zingiber officinale*	MSt	4% SA + MS + 2.5 mg/l BA	100 mM	53–66	Sundararaj et al. (2010)

(–) Not mentioned, *μM* micro molar, *AB* axillary bud, *ABA* abscisic acid, *AC* activated charcoal, *ApB* apical bud, *ASB* axial shoot bud, *B5* Gamborg medium (Gamborg et al. 1968), *BA* 6-benzyladenine, *BAP* 6-benzyl aminopurine, $CaCl_2$ calcium chloride, *cm* centimeter, *g* gram, *IAA* indole-3-acetic acid, *IBA* indole-3-butyric acid, *Kn* kinetin, *l* liter, *M* molar, *mg* milligram, *mM* millimolar, *MS* Murashige and Skoog (1962) medium (Murashige and Skoog 1962), *MR* microrhizome, *MSt* microshoots, *NAA* α-naphthalene acetic acid, *NS* nodal segment, P_{24} medium (Teasdale 1992), *PC* protocorm, *PG* phloroglucinol, *PLB* protocorm-like bodies, *ppm* parts per million, *PPM* plant preservative mixture™, *QL* Quoirin and Lepoivre medium (Quoirin and Lepoivre 1977), R4 medium-Chaleff and Stolarz (1981) medium, *S* sucrose, *SA* sodium alginate, *SB* shoot bud, *SE* somatic embryo, *ST* shoot tip, *TDZ* thidiazuron, *TrHR* transformed hairy root, *TrSB* transformed shoot bud, *WPM* woody plant medium (Lloyd and McCown 1980), *W/v* weight/volume, *Zea* zeatin

2.1.3 Nodal Segments

The nodal explants, obtained either from ex vitro/in vivo plants or in vitro plants, are also preferred propagules. However better plantlet recovery has been achieved using in vitro-derived nodal explants as compared to mature nodal segments obtained from field-grown plants. In medicinal plants, and as detailed in Table 1, varying degree of success has been achieved by encapsulating nodal explants in number of plants such as *Centella asiatica* (Prasad et al. 2014), *Ceropegia bulbosa* (Dhir and Shekhawat 2013), *Coleus forskohlii* (Swaroopa et al. 2007), *Decalepis hamiltonii* (Sharma and Shahzad 2012), *Mentha arvensis* (Islam and Bari 2012), *Ocimum basilicum* (Siddique and Anis 2009), *Pogostemon cablin* (Swamy et al. 2009), *Picrorhiza kurroa* (Mishra et al. 2011), *Rauvolfia serpentina* (Faisal et al. 2012), *Rauvolfia tetraphylla* (Alatar and Faisal 2012; Faisal et al. 2013), *Tylophora indica* (Faisal and Anis 2007), and *Vitex negundo* (Ahmad and Anis 2010). In many species, regeneration of one or more shoots from the synthetic seeds proved nodal segment encapsulation to be a viable strategy.

As apparent from literature, among various unipolar propagules, nodal segments are the most suitable for encapsulation studies due to presence of generally two pre-existing axillary meristems compared to one in shoot tip explants. One of the limitations faced is the inability of shoots to form roots in vitro especially for recalcitrant RET plants and woody plant species. On the other hand, in *Coleus forskohlii* and *Picrorhiza kurroa*, encapsulated nodal segments produced roots on PGR-free nutrient media (Mishra et al. 2011; Swaroopa et al. 2007). However, in some species, it was required to include a step for root induction in the encapsulation protocols. It involves either incubating explants, before encapsulation, in dark to induce root primordia, or inclusion of PGR to the encapsulation matrix or regrowth media (Sharma et al. 2013).

2.1.4 In Vitro Storage Organs

Storage organs such as bulbs, rhizomes, and protocorms are natural propagules that can also be induced under in vitro conditions. Despite being a viable and promising option, there are hardly any reports regarding encapsulation of these explants in medicinal plants. In *Allium sativum* a protocol for the conservation using encapsulated in vitro-induced bulblets with good shoot recovery (6.30), on MS + BA (2 mg/l) + NAA (2 mg/l), has been established (Bekheet 2006). In *Acorus calamus* storage of encapsulated microrhizome for 24–48 weeks also could be achieved (Quraishi et al. 2017).

2.2 Encapsulation Matrix

In synthetic seed technology, the encapsulating agent serves as a coating around the explant that includes mineral elements, source of carbohydrate (generally sucrose),

growth regulators, etc. in water or standard nutrient media (MS, B5, WPM etc.) (see Table 1) and acts as an artificial endosperm. Hence it has a significant role on the ultimate sustainability of the synthetic seed. The "synthetic seed coat" (insoluble gel matrix of calcium alginate) provides nutrients for growth and shields the explants during storage and handling. Selection of plant material is equally important. Thus, the crucial factors responsible for the successful synthetic seeds include the concentration and type of gel required for encapsulation and the duration of exposure of beads to calcium chloride ($CaCl_2$) (Redenbaugh 1993).

During the initial phase, much of the research was directed towards selection of suitable encapsulation matrix and combinations of chemicals. Sodium alginate (SA) has been advocated by various researchers owing to its moderate viscosity, non-toxicity, low cost, the long-term storability, quick gelation properties, and hardening of beads at room temperature (Kumari et al. 2014). The variation in Na-alginate concentration for alginate bead formation in different plant species (Table 1) may be attributed to the use of different brands or batch-to-batch variation within same brand and not specific to the plant species (Mandal et al. 2000; Sharma et al. 2009b).

In medicinal plants also SA (3–6%) with 75–125 mM $CaCl_2$ has been used in various laboratories in India. Detailed experiments conducted in a number of medicinal plants at Central Institute of Medicinal and Aromatic Plants at Lucknow, India, indicated that though use of SA from various batches affected bead quality, concentration of SA required appropriate adjustment, but the beads sprouting remained unaffected. However the $CaCl_2$ solution concentration remained the same (75 mM) (Ahuja et al. 1989).

In authors' laboratory, a combination of 3% SA (Sigma) with 100 mM $CaCl_2$ (Hi Media) has resulted in high-quality bead formation in a number of plants such as *Allium* spp., *Bacopa monnieri*, *Curculigo orchioides*, *Pogostemon patchouli*, *Zingiber officinale*, etc. (Fig. 1a, b).

In *Swertia chirayita*, encapsulation of somatic embryos using 4% SA and 100 mM $CaCl_2$ proved best for obtaining uniform high-quality beads (Kumari et al. 2014). Similarly the use of 4% SA was optimum for firm and uniform beads in *Curcuma longa*, *Decalepis hamiltonii* (Sharma and Shahzad 2012), *Pogostemon cablin* (Swamy et al. 2009), and *Zingiber officinale* (Gayatri et al. 2005; Sharma et al. 1994).

Duration of complexation of SA with $CaCl_2$ also had a bearing on quality of beads. Longer complexation durations adversely affected the percent plantlet development from encapsulated shoot buds (Ahuja et al. 1989). Duration of 30 min has been found sufficient for complete complexation.

It was evident from the aforementioned information that the majority of the reports employed 3% SA and 100 mM $CaCl_2$ to facilitate proper ion exchange which thus produces the most advantageous bead formation (Gantait et al. 2015). Beads produced with lower levels of SA (1–2%) were asymmetrical in shape, remarkably fragile, and soft to handle, while those with 5% or higher SA were too rigid for regrowth and germination of explants (Gantait et al. 2015). This combination has been successfully employed for all propagules including somatic embryos, shoot tip/shoot bud, and nodal segments, for instance, in *Rauvolfia serpentina* (Faisal et al. 2012), *Rauvolfia tetraphylla* (Faisal et al. 2013), *Tylophora indica* (Faisal and Anis 2007), *Vitex* negundo (Ahmad and Anis 2010), etc., and the regeneration of synthetic seeds was ~90%.

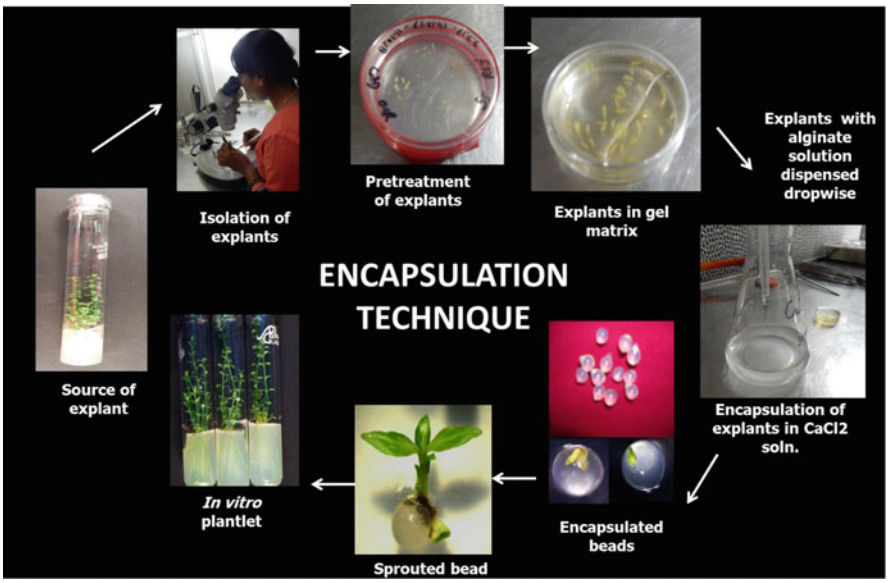

Fig. 1 Flow chart showing steps of encapsulation techniques in *Bacopa monnieri*

Regarding the type of carbohydrate, sucrose has been used most commonly as component of encapsulation matrix. In many cases, addition of growth regulators to improve germination and/ or induce rooting has been beneficial (Table 1, Gantait et al. 2015; Sharma et al. 2013). Protective chemicals such as antibiotics may be added to protect the synthetic seed from fungal and/or bacterial infection especially if the sowing is to be done on soil/soilrite (Ahuja et al. 1989).

2.3 Regrowth

For effective utilization, encapsulated explants are regenerated in vitro, using defined basal media (MS/ WPM) at 1/2, 1/3, 1/4, or full-strength (Lloyd and McCown 1980) or ex vitro directly on soil or any substrate. Conversion to plantlets has been the most crucial aspect of synseed technology that limits the commercial exploitation of this technology. Availability of appropriate nutrient to encapsulated propagule is the crucial factor for its germination (Mallikarjuna et al. 2016). For somatic embryos, germination on solidified agar yields good results. In some cases, supplementation of plant growth regulators (PGRs) to the regrowth medium boosts the germination of artificial seeds. The inclusion of BA (2.5 mg/l) to the medium was beneficial for early initiation of regrowth of the synseeds. Addition of cytokinin has been a promising strategy for increasing the number of shoots/synseed without any adverse effects on shoot and root growth. For example, presence of cytokinins in medium with full strength of MS salts, e.g., kinetin for ginger (Sharma et al. 1994)

and BA + NAA for *Tylophora indica* (Faisal and Anis 2007) while in *Ocimum basilicum* half-strength MS with BA + IAA (Siddique and Anis 2009) resulted in improved proliferation of shoots developed from synseeds. The rate of germination is known to vary noticeably with different media concentrations (Ahuja et al. 1989). Ahmad and Anis (2010) reported beneficial effect of inclusion of Kn (2.5 μM) + NAA (1.0 μM) to MS for inducing roots in encapsulated nodal segments of *Vitex negundo*, while WPM + BA (7.5 μM) and NAA (2.5 μM) improved germination and plantlet formation in *Rauvolfia tetraphylla* (Alatar and Faisal 2012).

Among various propagules, shoot tips have exhibited high regrowth in majority of medicinal plants like *Picrorhiza kurroa* (Mishra et al. 2011), *Rauvolfia serpentina* (Ray and Bhattacharya 2008), *Eclipta alba* (Ray and Bhattacharya 2010), *Bacopa monnieri* (Sharma et al. 2016), and *Zingiber officinale* (Sundararaj et al. 2010) (Table 1).

There was improved regrowth of explants when encapsulation matrix had nutrients as compared to that without nutrient (Ahmad and Anis 2010; Chand and Singh 2004; Sundararaj et al. 2010). Supplementation of PGRs and growth additives was conducive for synseed conversion. In *Adhatoda vasica*, Anand and Bansal (2002) reported highest conversion using synseeds prepared in B5 medium containing kinetin (Kn) and phloroglucinol (PG) when cultured on B5 basal medium. However, reduced response was observed when regrowth medium was also supplemented Kn and PG irrespective of encapsulation matrix (i.e., with or without growth additives). Thus, the presence of nutrients and PGRs in the gel matrix seems more critical than that in the regrowth medium.

Direct planting of encapsulated propagules to soil or other substrates is expected to circumvent the acclimatization procedure required for in vitro-raised plantlets. As indicated above, synseed can be grown directly on soil/soilrite, etc. though substrata other than agar showed lower percent shoot emergence of *Picrorhiza kurroa* and *Valeriana wallichii* (Ahuja et al. 1989) (Table 2). Synseeds are, in general, prone to bacterial, fungal, and/or microbial infections especially during ex vitro regeneration even under controlled conditions (Vij and Kaur 1994). To control such infections, inclusion of antibiotics and antifungal agents to the gel matrix has been advocated (Ahuja et al. 1989; Sharma et al. 2013). In *Picrorhiza kurroa*, inclusion of tetracycline (100 mg/l) + bavistin (250 mg/l) resulted in delay in germination, reduced normal plantlet formation, and inhibition of bacterial and fungal growth. However, in many species such chemicals affect recovery which can be taken care of by including PGRs in the encapsulation matrix.

3 Application of Synseed Technology

Since the first successful report of encapsulation of non-embryogenic propagule (Bapat et al. 1987), the synseed technology has been extended to an array of plant species including medicinal plants (see Sharma et al. 2013). In medicinal plants, the technique has been successfully applied for the encapsulation of axillary and apical

Table 2 Comparison of in vitro and ex vitro regeneration of synthetic seeds in medicinal plants

Plant species	Propagule	Regeneration (%)		References
		In vitro	Ex vitro	
Althaea officinalis	NS	64.6	55	Naz et al. (2018)
Centaurium erythraea	ST, HR	56–86	90	Piatczak and Wysokińska (2013)
Ceropegia barnesii	NS	–	85–97	Ananthan et al. (2018)
Coleus forskohlii	ST, NS	75	61	Swaroopa et al. (2007)
Dioscorea bulbifera	NS	70–75	82	Narula et al. (2007)
Mandevilla moricandiana	NS	83.30	100	Cordeiro et al. (2014)
Nyctanthes arbor-tristis	NS	86.66	90	Jahan and Anis (2015)
Picrorhiza kurroa	SB	87	30	Ahuja et al. (1989)
Rauvolfia serpentina	ST	68.5–100	80	Ray and Bhattacharya (2008)
Rauvolfia tetraphylla	NS	90.3	90	Alatar and Faisal (2012)
Solanum nigrum	ST	97.2–100	55.5–94.40	Verma et al. (2010)
Spilanthes acmella	ST	73.60	90	Sharma et al. (2009b)
Valeriana wallichii	SB	98	64	Ahuja et al. (1989)
Zingiber officinale	MSt	100	53	Sundararaj et al. (2010)

HR hairy root, *MSt* microshoot, *NS* nodal segment, *SB* shoot bud, *ST* shoot tip

shoot buds of in vitro grown plants of >80 species including RET plants such as *Picrorhiza kurroa*, *Rheum emodi*, *Rauvolfia serpentina*, *Valeriana wallichii*, etc. Realizing its potential for the clonal propagation, recently, the technology has attracted the interest of scientific community for plant genetic resource management. The efficiency of the technique lies in small size of synseeds, ease of handling, and requirements of minimum space, time, and care (Mishra et al. 2011). Facilitation of easy ex vitro transplantation coupled with feasibility of ex vitro root induction offers added advantage especially for rehabilitation of plants under natural conditions. In ginger encapsulation of shoot buds has also been applied for producing disease-free planting material (Sharma et al. 1994). Further, synseeds greatly aid in the short- and long-term storage of propagules and germplasm exchange within and across countries (Germana et al. 2011). Besides conservation, ready availability of the propagules for propagation is also ensured (Ray and Bhattacharya 2008).

Synthetic seed technology has also been applied for regeneration of transgenic plants. In *Centaurium erythraea* and *Plumbago indica*, encapsulated, genetically transformed hairy root fragments and shoot buds could be successfully stored for 14 and 24 weeks, respectively, with high regeneration (Piatczak and Wysokińska 2013; Gangopadhyay et al. 2011). Regenerated, transformed plantlets after conservation exhibited retention of secondary metabolite, plumbagin, in *Plumbago indica* (Gangopadhyay et al. 2011).

3.1 Propagation of Medicinal Plants

Micropropagation through encapsulation is an efficient practice for mass propagation of the plant species investing minimum time and space. Beads can be grown in vitro, on regrowth medium, or ex vitro on substrates like gravel, perlite, sand, soil, soilrite, vermiculite, and vermicompost for regeneration into plantlets. Table 1 provides an exhaustive list of medicinal plants for which propagation has been attempted using synseed technology. It is evident that SA (3–4%) and $CaCl_2$ have been successful in majority of the cases. In some cases, inclusion of PGR in encapsulation matrix has improved regrowth of beads. Additionally, medium supplemented with PGR is more beneficial than PGR-free culture medium for successful conversion (Mandal et al. 2000). The requirement of growth regulators in culture medium varies from species to species. For example, artificial seeds of *Ocimum* spp. exhibited ~90% recovery on MS containing different concentrations of BA—1.1 μM in *O. americanum*, 4.4 μM in *O. basilicum* and *O. sanctum*, and 2.2 μM in *O. gratissimum* (Mandal et al. 2000). In contrast, artificial seeds of *Zingiber officinale* (Sundararaj et al. 2010) exhibited highest conversion on PGR-free MS medium (irrespective of its strength). Agar has been the common gelling agent for regrowth medium (Cameron 2008). According to the reports available in literature, encapsulation technique has been applied using shoot tips, nodal segments, and/or apical shoot buds of in vitro grown plants in >80 medicinal plants (Table 1). Although the regrowth response varied from species to species, high germination of encapsulated explants (70–90%) has been achieved in a large number of species (Table 1).

With distinct advantages, the economy of cost owing to reduced requirement of space, culture medium, and time, synseed is a powerful low-cost tool for mass propagation of elite species with high medicinal value and especially for rare or endangered taxa. Coupled with the feasibility of direct ex vitro transfer, it gains special significance for species for which simulation of conditions for acclimatization is extremely challenging.

Successful ex vitro transfer of encapsulated propagules on different planting substrates has been reported only for a few medicinal plant species (Ahuja et al. 1989), and a comparison of regeneration in a controlled culture room environment and that under greenhouse conditions is presented in Table 2. The limiting factor noticed for reduced regrowth rate under ex vitro conditions is the unavailability of optimum nutrients and greater susceptibility to contamination. As a result, it is indispensable to maintain a nutrient pool for the encapsulated propagules. Attempts to directly sow the encapsulated beads using different propagules under ex vitro conditions in ~15 medicinal plants (see Table 2) resulted in >50% germination in all except *P. kurroa*. However inclusion of antibiotics and fungicides in the encapsulation matrix improved germination in *P. kurroa* under glasshouse conditions (Ahuja et al. 1989). Some researchers added MS nutrients at 1/4 or 1/2 strength to soilrite for improved ex vitro regeneration of artificial seeds with 62 and 43% regeneration in *Phyllanthus amarus* and *T. indica*, respectively (Singh et al. 2006a; Faisal and Anis 2007).

3.2 Rehabilitation of Medicinal Plants

Unsystematic harvesting of medicinal plants from nature coupled with endemism, limited population size, and destruction of natural habitat have led to the decline of a large number of medicinal plants. Thus, many plants have become rare, threatened, and endangered. Propagation and restoration using synthetic seed technology can be viewed as a potential option to conserve RET species. There are only few reports on restoration of plants using in vitro propagation technique per se (Sharma et al. 2014), whereas using synthetic seeds, there are only two reports on *Ceropegia barnesii* (Ananthan et al. 2018) and *Ipsea malabarica* (Martin 2003b).

In *Ceropegia barnesii*, an endemic and endangered plant, Ananthan and coworkers standardized in vitro regeneration and synthetic seeds technology with an aim to support restoration in the wild. Optimum multiple shoot formation from synthetic seeds was achieved on MS + BA + GA3. Following root induction, inoculation of vesicular-arbuscular mycorrhizal fungi (*Glomus aggregatum* and *G. intraradices*) on the hardening trials enhanced the survival rate during hardening. The regenerated plants were reintroduced to their natural habitat for further experiments (Ananthan et al. 2018).

3.3 In Vitro Conservation

In recent years, cost-effectiveness and simplicity of synseeds technologies using vegetative propagules makes it an easier and popular option for not only propagation but also for germplasm conservation (Islam and Bari 2012; Remya et al. 2013; Sharma et al. 2016). Alginate encapsulation greatly aids in the short- and long-term conservation due to avoidance of use of harmful chemicals. Besides ensuring ready availability, it also facilitates germplasm exchange (Rao et al. 1998; Naik and Chand 2006). Additional advantages for conservation include small size, ease of handling and transportation, and retention of genetic stability even after cryopreservation (Ray and Bhattacharya 2008; Mishra et al. 2011)

3.3.1 Short-Term Conservation

In most of the procedures, the mandatory step is germination or regrowth of encapsulated propagule and further transfer out. One of the special features for conservation is the uniform coating of propagules coupled with small size leading to ease of handling individual bead like a seed. Thus the basic objective of increasing the shelf life of propagule can be achieved by desiccation of beads, quite akin to seeds. It is important to note that using synthetic seeds, short-term conservation for few weeks can be achieved in comparison to medium-term conservation using in vitro shoot cultures. The strategies adopted for synseed conservation include:

- Incubation at different temperatures
- Desiccation using air or osmotica like sucrose
- Storage in a cryovial without any nutrient media

Regarding short-term conservation of medicinal plants using one or combination of strategies, details are presented in Table 3. The following is a brief account of successful short-term conservation of selected medicinal plants and the strategies adopted:

In some of the medicinal plants, 4 °C is suitable for short-term storage of encapsulated beads (Faisal and Anis 2007; Sharma et al. 2009a, b; Ahmad and Anis 2010; Tabassum et al. 2010). However, the temperature requirement for optimum storage differs from species to species. Storage in dark at 25 °C has been reported beneficial for tropical and subtropical species as these cannot tolerate low temperature (Table 3).

In author's laboratory, among various temperatures (4, 10, 15, 20, and 25 °C) tested on conservation of encapsulated shoot tips of *Bacopa monnieri* conservation in a cryovial without nutrient medium, 25 °C was the most suitable temperature with 6 months conservation period. While those stored at 4 °C lost viability within 1 week (Sharma et al. 2016). In contrast, Muthiah et al. (2013) reported 4 °C to be promising for encapsulated shoot tips of *Bacopa monnieri*, and the beads stored at 24 °C started sprouting within a week. This may be due to difference in method of storage adopted.

In *Rauvolfia serpentina*, beads stored at three different temperatures (20, 12, and 4 °C) indicated 4 °C to be the most suitable with (68.5–100%) regrowth up to 14 weeks. However, sharp loss of viability was observed after 14 weeks. The synseed-derived plantlets exhibited 80% ex vitro establishment after root induction and acclimatization (Ray and Bhattacharya 2008). Low temperature (4 °C) has also been reported to be beneficial for storage in *Spilanthes acmella* (Singh et al. 2009) and *Eclipta alba* (Ray and Bhattacharya 2010).

Sundararaj et al. (2010) reported that encapsulated microshoots of *Zingiber officinale* conserved at 25 °C exhibited 100% regrowth, while there was no regrowth in those stored at 4 °C in dark. Similarly, in *Cineraria maritime* and *Picrorhiza kurroa*, 25 °C proved suitable for conserving synthetic seeds for 6 and 3 months, respectively (Srivastava et al. 2009; Mishra et al. 2011).

At the in vitro genebank at NBPGR, India experiments conducted on short-term conservation of ginger germplasm revealed better storage of synseeds after sucrose dehydration in comparison to that after air dehydration or fresh synseeds. Following sucrose dehydration (0.25 M sucrose for 16 h) and storage at 25 °C, beads exhibited 53 and 13% conversion after 8 and 12 weeks, respectively (Sundararaj et al. 2010).

In *Bacopa monnieri* shoot tip-derived synseeds exhibited better regrowth (~86%) as compared to encapsulated nodal segments (60%) after 6 months of storage (Muthiah et al. 2013). However, in *Coleus forskohlii* and *Picrorhiza kurroa*, both shoot tip- and nodal segment-derived synthetic seeds exhibited similar regrowth after 24 and 12 weeks of storage (Swaroopa et al. 2007; Mishra et al. 2011).

Retention of germination or sprouting potential even after an adequate period of storage is an essential aspect. Synthetic seeds (synseeds) produced through encapsulation technique are capable of producing an entire plant after storage at room

Table 3 Application of synthetic seed technology for in vitro short-term conservation of medicinal plants

Species	Explant	Storage duration	Storage conditions	Regeneration (%)	References
Acorus calamus	MR	24 weeks	10 °C + dark	100	Quraishi et al. (2017)
Acorus calamus	MR	48 weeks	10 °C + dark	80	Quraishi et al. (2017)
Allium tuberosum	Shoot bases	8 weeks	25 °C;16 h/8 h photoperiod, in a cryovial without any nutrient medium	–	Anonymous (2004)
Althaea officinalis	NS	4 weeks	4 °C	64.60	Naz et al. (2018)
Bacopa monnieri	ST	24 weeks	4 °C	86.60	Muthiah et al. (2013)
Bacopa monnieri	NS	24 weeks	4 °C	60	Muthiah et al. (2013)
Bacopa monnieri	ST	24 weeks	25 °C; 16h/8h photoperiod, in a cryovial without any nutrient medium	80–100	Sharma et al. (2016)
Catharanthus roseus	SE	8 weeks	4 °C	81.67	Maqsood et al. (2012)
Cineraria maritime	MSt	24 weeks	25 ± 2 °C	11.76	Srivastava et al. (2009)
Centaurium erythraea	HR	6 weeks	4 °C	86	Piatczak and Wysokińska (2013)
Centaurium erythraea	SB	6 weeks	4 °C	56	Piatczak and Wysokińska (2013)
Ceropegia bulbosa	NS	8 weeks	4 °C	50.70	Dhir and Shekhawat (2013)
Cineraria maritime	ST, NS	24 weeks	25 ± 2 °C	82	Srivastava et al. (2009)
Clitoria ternatea	SE	20 weeks	25 ± 2 °C	92	Kumar and Thomas (2012)
Coleus forskohlii	ST, NS	24 weeks	4 °C	75	Swaroopa et al. (2007)
Decalepis hamiltonii	NS	8 weeks	4 °C	77	Sharma and Shahzad (2012)
Dioscorea bulbifera	NS	4 weeks	4 °C	75	Narula et al. (2007)
Dioscorea bulbifera	NS	5 weeks	4 °C	70	Narula et al. (2007)

(continued)

Table 3 (continued)

Species	Explant	Storage duration	Storage conditions	Regeneration (%)	References
Eclipta alba	NS	8 weeks	4 °C	51.20	Ray and Bhattacharya (2010)
Glochidion velutinum	ST	4 weeks	4 °C	86.40	Mallikarjuna et al. (2016)
Hibiscus moscheutos	NS	78 weeks	5 °C	80	West et al. (2006)
Mandevilla moricandiana	NS	4 weeks	15 °C	33.3	Cordeiro et al. (2014)
Mentha arvensis	NS	8 weeks	4 ± 1 °C	44–46	Islam and Bari (2012)
Nyctanthes arbor-tristis	NS	3 weeks	4 °C	86.66	Jahan and Anis (2015)
Ocimum americanum	AB	8 weeks	4 °C	31	Mandal et al. (2000)
Ocimum basilicum	AB	8 weeks	4 °C	22	Mandal et al. (2000)
Ocimum gratissimum	AB	8 weeks	4 °C	13	Mandal et al. (2000)
Ocimum sanctum	AB	8 weeks	4 °C	18	Mandal et al. (2000)
Phyllanthus amarus	ST	8 weeks	4 °C	47	Singh et al. (2006a)
Picrorhiza kurroa	ST, NS	12 weeks	25 ± 2 °C	21.43	Mishra et al. (2011)
Rauvolfia serpentina	ST	18 weeks	4 °C	68.5–87.50	Ray and Bhattacharya (2008)
Rauvolfia serpentina	NS	4 weeks	4 °C	80	Faisal et al. (2012)
Rauvolfia tetraphylla	NS	4 weeks	4 °C	90.30	Alatar and Faisal (2012)
Rauvolfia tetraphylla	NS	4 weeks	4 °C	90	Faisal et al. (2013)
Rhinacanthus nasutus	SE	45 days	25 ± 2 °C	94	Cheruvathur et al. (2013b)
Rumex vesicarius	SE	45 days	4 °C	85	Nandini et al. (2014)
Salvia officinalis	ST	6 weeks	4 °C	88	Grzegorczyk and Wysokińska (2011)
Salvia officinalis	ST	24 weeks	4 °C	63	Grzegorczyk and Wysokińska (2011)

(continued)

Table 3 (continued)

Species	Explant	Storage duration	Storage conditions	Regeneration (%)	References
Solanum nigrum	ST	8 weeks	4 °C	25	Verma et al. (2010)
Spilanthes mauritiana	NS	3 weeks	4 °C	>90	Sharma et al. (2009a)
Spilanthes acmella	ST	8 weeks	4 °C	50	Singh et al. (2009)
Spilanthes acmella	NS	6 weeks	4 °C	73.60	Sharma et al. (2009b)
Tylophora indica	NS	45 days	15 ± 1 °C	70	Gantait et al. (2017b)
Withania coagulans	ST, NS	8 weeks	4 °C	72	Rathore and Kheni (2015)
Zingiber officinale	MSt	8 weeks	25 °C + dark	53	Sundararaj et al. (2010)

AB axillary bud, *HR* hairy root, *MR* microrhizome, *MSt* microshoot, *NS* nodal segment, *SB* shoot bud, *SE* somatic embryo, *ST* shoot tip

temperature or under low temperatures, above 0 °C (Gantait et al. 2015). As evident from Table 3, majority of medicinal plants exhibited high regrowth, on transfer to suitable medium and developed into normal shoots (Fig. 2c–e). For example, very high regrowth was achieved in *Glycyrrhiza glabra* (98%) (Mehrotra et al. 2012) and in *Cineraria maritime* (82%) (Srivastava et al. 2009) following 6 months of storage.

3.3.2 Long-Term Conservation

Cryopreservation, the only available long-term conservation option, is applicable subject to avoidance of intracellular ice crystal formation which results in irreparable damage to living cells. In recent years, based on encapsulation technology, new cryopreservation techniques, encapsulation-dehydration and encapsulation-vitrification, have been developed.

The approach of encapsulation-dehydration technique, developed for *Solanum* shoot tips by (Fabre and Dereuddre 1990), is more handy and does not require either a costly programmable freezer or harmful cryoprotectants (Reinhoud et al. 2000). The method is based on successive osmotic and air desiccation of plant cells allowing gradual removal of water from encapsulated propagules in sucrose-rich medium. Air-drying or desiccation in a laminar air flow further increases sucrose concentration in the beads, thus preventing ice crystal formation during freezing to −196 °C in liquid nitrogen (Engelmann 2000). In encapsulation-dehydration method, the steps include encapsulation of isolated shoot tips in alginate beads, dehydration in high sucrose-supplemented liquid medium (0.5–0.8 M) for 1–7 days, partial desiccation in the air current of a laminar air flow cabinet or with silica gel, rapid freezing, thawing, and regrowth (Engleman 2003) (Fig. 3).

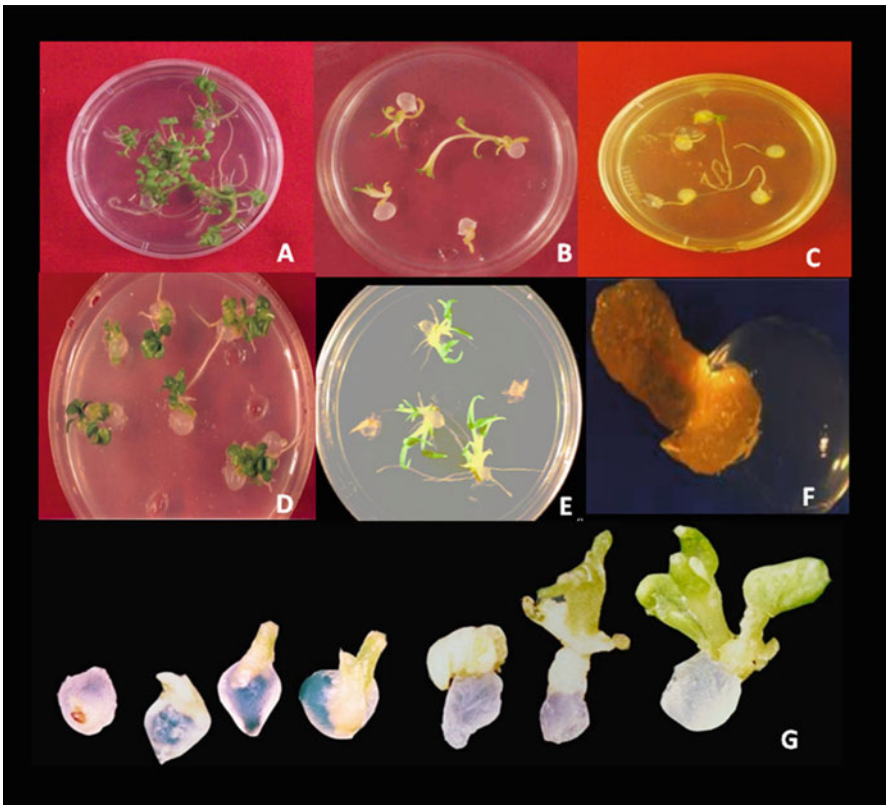

Fig. 2 Regeneration of encapsulated shoot tips (prepared using 3% SA and 100 mM calcium chloride) in *Bacopa monnieri* (**a**) and *Gentiana kurroo* (**b**); regrowth of synthetic seeds after short-term conservation in *Allium tuberosum* (**c**), *Bacopa monnieri* (**d**), and *Zingiber officinale* (**e**); and regrowth of encapsulated shoot tips after cryopreservation in *Dioscorea bulbifera* (**f**) and *Holostemma annulare* (**g**)

The encapsulation-vitrification is a hybrid of the two techniques—encapsulation dehydration and vitrification—that reduces any potential injury from vitrification (Sakai 2000) and offers various advantages of better recovery over encapsulation-dehydration (Wang et al. 2004). Encapsulation-vitrification method involves encapsulation of explants followed by vitrification using cryoprotectant solution, freezing, thawing, and regrowth (Fig. 4). Embryogenic callus of *Dioscorea bulbifera* was successfully cryopreserved using encapsulation-vitrification (Yin and Hong 2010).

Cryopreservation of medicinal plants has received attention only in the past decade and is still at an experimental stage (Sharma and Pandey 2013, 2018). It is encouraging to note that even these new techniques—ED and EV—have been reported in ~10 medicinal plants with limited success (Table 4). Encapsulation-dehydration technique resulted in high-frequency regeneration from cryopreserved explants of *Dioscorea* spp. (Mandal 2000, 2003; Mandal and Ahuja-Ghosh 2007) and in *Holostemma annulare* (Decruse et al. 1999, 2004) (Fig. 2f, g). Following

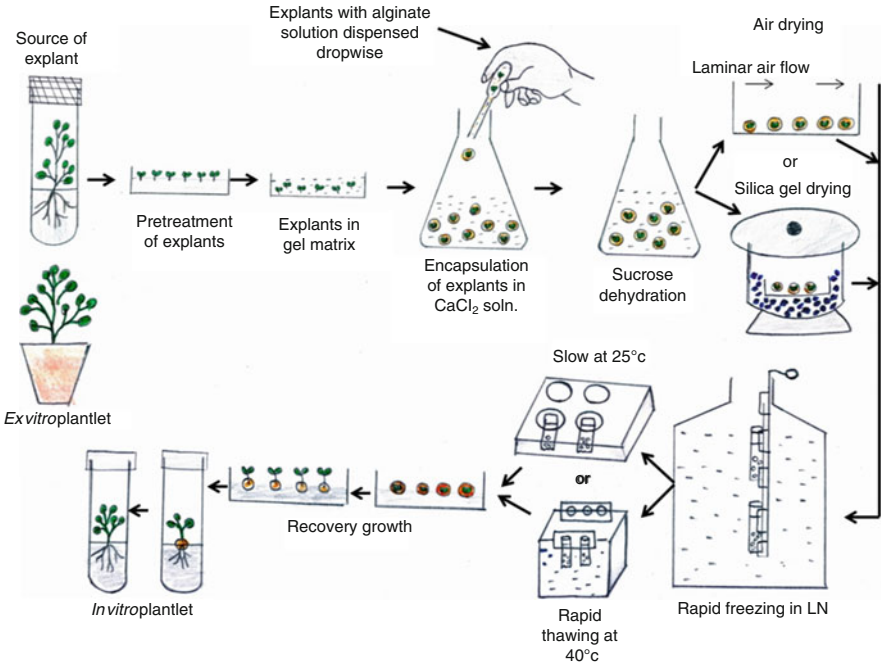

Fig. 3 Diagrammatic representation of cryopreservation of explants using encapsulation-dehydration (ED) technique

detailed investigations, Decruse and coworkers opined that during preparative procedures of ED, removal of NH+ ions (ammonium nitrate) from culture medium improved post-thaw recovery in *Holostemma annulare* (Decruse et al. 1999, 2004; Decruse and Seeni 2002).

In our laboratory preliminary success has been achieved in *Allium tuberosum*, *A. sativum*, *Bacopa monnieri*, and *Rauvolfia serpentina* using ED with plantlet recovery after cryopreservation (unpublished data). More research is required for applying these methods for germplasm conservation in the genebank, as the methods are known to be genotype specific (Sharma et al. 2017).

Using EV, successful cryopreservation of shoot tips has been reported in two medicinal plants, namely, *Artemisia herba-alba* and *Astragalus membranaceus* (Sharaf et al. 2012; Ming-Hua and Sen-Rong 2015). It is important that using shoot tips, EV resulted in higher post-thaw recovery as compared to ED (Sharaf et al. 2012).

3.4 Germplasm Exchange

Synthetic seed technology has been advocated as a promising option for facilitating exchange of germplasm. For effective exchange, viability of encapsulated explant during transit is a prerequisite to ensure successful regrowth after transportation (Hasan and Takagi 1995).

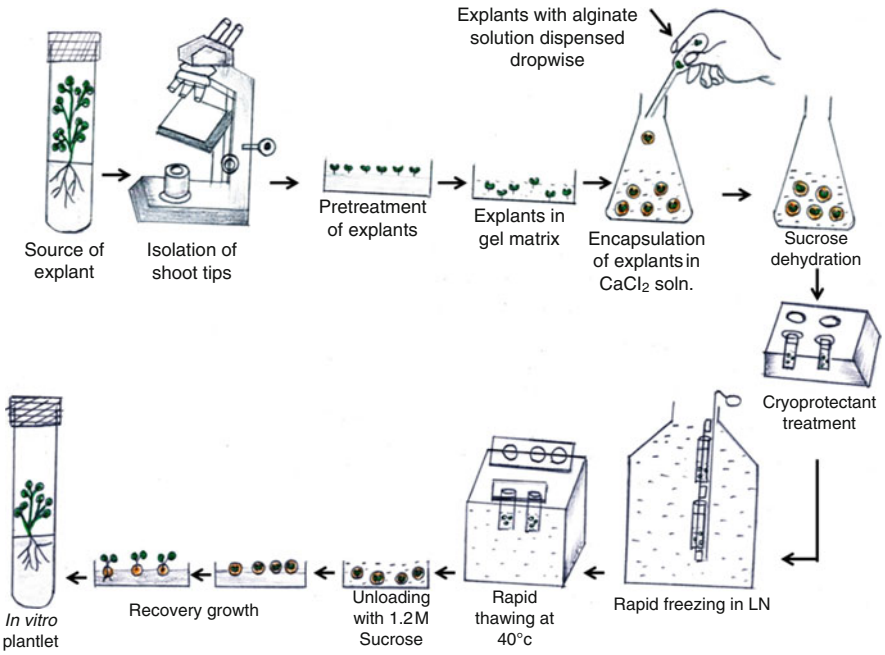

Fig. 4 Schematic representation of encapsulation-vitrification (EV) technique for cryopreservation

For this purpose, work has been carried out in a large number of medicinal plants to optimize storage conditions resulting in high regrowth (Table 3). As indicated in Sect. 7.3.3.1, low temperature (4 °C) has been suitable for synseed storage in some species (Faisal and Anis 2007; Sharma et al. 2009a, b; Singh et al. 2007), whereas in a number of tropical plants, the suitable temperature is 25 °C (Sharma et al. 2016; Sundararaj et al. 2010).

Besides successful short-term conservation of beads with high regrowth after 8–12 weeks, the added advantage of storage at 25 °C in dark (24 h) is simulation of the transit conditions during germplasm exchange. Further, method of storing synseeds in a cryovial without nutrient medium developed in our laboratory for *Bacopa monnieri* (Sharma et al. 2016) and *Allium sativa*, *A. tuberosum*, and *Kaempferia* spp. (Unpublished data) has the potential for international exchange due to reduced quarantine issues without the risk of contamination during transit due to avoidance of nutrient medium in the vial.

Retention of high percentage of germination or sprouting potential even after >8 weeks of storage (Table 3) is an essential prerequisite for application of synseed technology for germplasm exchange purpose. As 8 weeks is sufficient period of transport of material to any place, the technology could be effectively used specially for exchange of vegetative material without much quarantine restrictions.

Table 4 Application of synthetic seed technology for cryopreservation of medicinal plants

Species	Explant cryopreserved	Cryopreservation technique	Regeneration %	References
Allium sativum	Shoot tip	Encapsulation-dehydration	–	Anonymous (2011)
Allium tuberosum	Shoot tip	Encapsulation-dehydration	–	Anonymous (2014)
Artemisia herba-alba	Shoot tip	Encapsulation-dehydration	40	Sharaf et al. (2012)
Artemisia herba-alba	Shoot tip	Encapsulation-vitrification	68	Sharaf et al. (2012)
Astragalus membranaceus	Shoot tip	Encapsulation-dehydration	50	Ming-Hua and Sen-Rong (2015)
Astragalus membranaceus	Shoot tip	Encapsulation-vitrification	80	Ming-Hua and Sen-rong (2015)
Bacopa monnieri	Shoot tip	Encapsulation-dehydration	–	Sharma et al. (2014) and Sharma and Pandey (2018)
Gentiana tibetica	Cell suspensions	Encapsulation-dehydration	68	Mikuła et al. (2008)
Gentiana cruciata	Cell suspensions	Encapsulation-dehydration	83	Mikuła et al. (2008)
Gentiana kurroo	Shoot tip	Encapsulation-vitrification	–	Anonymous (2007) and Sharma and Pandey (2015)
Piper nigrum	Somatic embryo	Encapsulation-dehydration	62	Babu et al. (2012)
Dioscorea alata	Shoot tip	Encapsulation-dehydration	20	Malaurie et al. (1998)
D. bulbifera	Shoot tip	Encapsulation-dehydration	67	Mukherjee et al. (2009)
D. bulbifera	Shoot tip	Encapsulation-dehydration	60	Malaurie et al. (1998)
D. bulbifera	SE/embryogenic tissue	Encapsulation-dehydration	Regeneration of plants	Mandal (1999) and Mandal et al. (1996, 2009)
D. deltoidea	Shoot tip	Encapsulation-dehydration	76	Mandal and Dixit-Sharma (2007)
D. deltoidea	Shoot tip	Encapsulation-dehydration	Shoot regeneration	Mandal (2003) and Mandal and Dixit (2000)
D. floribunda	Shoot tip	Encapsulation-dehydration	75% survival; 25% regeneration	Mandal et al. (1996, 2000) and Mandal and Ghosh-Ahuja (2007)
Holostemma annulare	Shoot tip	Pregrowth; encapsulation-dehydration	54.2%	Decruse and Seeni (2002) and Decruse et al. (1999, 2004)
Ziziphora tenuior	Shoot tip	Encapsulation-dehydration	40	Baba et al. (2015)
Ziziphora tenuior	Shoot tip	Encapsulation-vitrification	37.5	Baba et al. (2015)

3.5 *Stability of Conserved Germplasm*

Shoot tips and shoot buds, also referred as microcuttings, have been the propagules of choice for encapsulation due to relative ease of availability of material once the in vitro propagation has been established (Piccioni and Standardi 1995; Standardi and Piccioni 1998). In most reports, a 3–5 mm-long single nodal segment, shoot tip, or shoot buds explants have been used for synseed production (Ahmad and Anis 2010). As shown in Table 1, there has been high rate of regeneration with encapsulated explants in ~80 medicinal plant species, but there is limited information on genetic stability of plantlets obtained from beads. Shoot tips and nodal segments, the preferred explants for synseed in case of medicinal plants, are known to exhibit a high degree of genetic stability (Piccioni 1997). However, increasing thrust on use of synthetic seeds for conservation and propagation necessitates genetic stability assessment following their conservation (Dehmer 2005).

Table 5 details the research carried out on aspects related to monitoring of genetic stability in medicinal plants using morphological, cytological, molecular, and/or biochemical analyses. Plantlets obtained from stored synseeds in *Decalepis hamiltonii* and ginger were morphologically similar to their mother plants (Sharma and Shahzad 2012; Sundararaj et al. 2010). In recent years, Bekheet (2006) and Mishra et al. (2011) used RAPD analysis to assess the genetic stability of synseed-derived plantlets of *Allium sativum* (garlic) and *Picrorhiza kurroa*, respectively. Using 45 RAPD markers, in *Picrorhiza kurroa* Mishra et al. (2011) confirmed genetic stability of plants derived from encapsulated microshoots following 3 months of storage. In *Rauvolfia serpentina* and *Rauvolfia tetraphylla* (Alatar and Faisal 2012; Faisal et al. 2012), genetic fidelity of plantlets after storage of encapsulated shoot tips was confirmed using 20 RAPD and 5 ISSR primers. Similarly in *Ceropegia bulbosa*, plants regenerated were morphologically and genetically identical based on RAPD analysis (Dhir and Shekhawat 2013). In *Dioscorea* spp. plantlets regenerated from cryopreserved shoot tips and embryogenic tissue using ED exhibited maintenance of genetic stability (Ahuja et al. 2002; Dixit et al. 2003; Dixit-Sharma et al. 2005)

In medicinal plants monitoring of active principle is the best but not always a feasible strategy owing to diverse nature of plants and the active principle (secondary metabolites), coupled with limited work on active principle analyses. In *Dioscorea* spp. plants regenerated from cryopreserved explants exhibited maintenance of stability based on biochemical (diosgenin) analysis (Dixit et al. 2003; Dixit-Sharma et al. 2005; Ahuja et al. 2002). Interestingly, in *Plumbago indica*, plantlets regenerated from encapsulated hairy root fragments exhibited maintenance of plumbagin content following 6 months of storage (Gangopadhyay et al. 2011).

4 Challenges and Future Prospects

Restoration and propagation using synthetic seed technology offer the possibility of conserving RET species with medicinal value. Successful conservation in genebanks using in vitro techniques is well documented and with increasing thrust on

Table 5 Genetic stability studies of plants regenerated after propagation and/or conservation using synthetic seed technology in medicinal plants

Plant species	Culture system	Strategy	Response	References
Bacopa monnieri	Regenerated plantlets from encapsulated shoot tips	Molecular (RAPD, ISSR), biochemical (HPLC) analysis	No variation was observed between control plants and in vitro regenerated plants after 6 months storage	Muthiah et al. (2013)
Bacopa monnieri	Alginate-encapsulated shoot tips	Molecular (RAPD) analysis	No variation between control plants and in vitro-regenerated plants	Rency et al. (2017)
Cannabis sativa	Encapsulated nodal segments	Molecular (ISSR) analysis	Genetic stability of plants derived from encapsulated microshoots following 3 months of storage	Lata et al. (2009b, 2011)
		GC-FID analysis	Showed homogeneity in the regrown plants and mother plants	
Dioscorea bulbifera	Synseed-derived plantlets	Molecular (RAPD) analysis	No variation observed	Narula et al. (2007)
Dioscorea bulbifera	Plants regenerated from cryopreserved encapsulated embryogenic tissue	Molecular (RAPD), biochemical (HPLC), and morphological analyses	Cryopreserved-derived plants maintained genetic stability	Dixit et al. (2003)
Dioscorea deltoidea	Cryopreserved shoot tip-derived in vitro plantlets	Biochemical (HPLC) and morphological analyses	Cryopreserved-derived plants maintained genetic stability	Dixit-Sharma et al. (2005)
Dioscorea floribunda	Plants regenerated from cryopreserved encapsulated embryogenic tissue	Molecular (RAPD), biochemical (HPLC), and morphological analyses	Cryopreserved-derived plants maintained genetic stability	Ahuja et al. (2002)
Eclipta alba	Encapsulated microshoots	Molecular (RAPD) analysis	No variation was observed	Ray and Bhattacharya (2010)
Gentiana tibetica	Encapsulated cell suspensions	Flow cytometry	Cryopreservation did not influence the genome size either in PEM or in regenerants	Mikuła et al. (2008)
G. cruciata	Encapsulated cell suspensions	Flow cytometry	Cryopreservation did not influence the genome size either in PEM or in regenerants	Mikuła et al. (2008)

(continued)

Table 5 (continued)

Plant species	Culture system	Strategy	Response	References
Glycyrrhiza glabra	Synseed-derived plantlets	Molecular (RAPD and ISSR) analysis	No significant differences observed in plants derived from alginate-encapsulated microshoots and parent material	Mehrotra et al. (2012)
Picrorhiza kurroa	Synseed-derived plantlets	Molecular (RAPD) analysis	Genetic stability of plants derived from encapsulated microshoots following 3 months of storage	Mishra et al. (2011)
Picrorhiza kurroa	Synseed-derived plantlets	Molecular (RAPD and ISSR) analysis	No significant differences observed in regenerants and mother plant, but notable differences observed among three adventitious shoots regenerated from three calli	Rawat et al. (2013)
		Phytochemical study (HPLC)	Tissue culture-raised plants showed higher secondary metabolite (picrotin and picrotoxinin) as compared to mother plant	
Rauvolfia serpentina	Synseed-derived plantlets	Molecular (RAPD and ISSR) analysis	Genetic stability of plants derived from encapsulated microshoots after storage	Faisal et al. (2012)
Rauvolfia tetraphylla	Synseed-derived plantlets	Molecular (RAPD and ISSR) analysis	No change in the RAPD and ISSR profiles among the regenerated plantlets	Alatar and Faisal (2012)
Withania somnifera	Synseed-derived plantlets	Molecular (RAPD and ISSR) analysis	No variation was observed	Fatima et al. (2013)

GC-FID gas chromatography (GC)-flame ionization detector (FID) analysis, *HPLC* high-performance liquid chromatography, *ISSR* inter-simple sequence repeat, *PEM* proembryogenic mass, *RAPD* random amplified polymorphic DNA

application of synthetic seed technology for propagation coupled with advances in cryopreservation, methods are predicted to boost this further in case of medicinal plants.

In medicinal plants, there are limited reports of somatic embryogenesis with few reports on conversion of somatic embryos, and these are the factors limiting the

application of this technology. While encapsulation of shoot tips and shoot buds ensures high germination and stability of conserved synseeds, in vitro rooting is the major obstacle in many species, including recalcitrant MAPs and RET plants. Thus, there is a need to work out a strategy to facilitate its use for large-scale utilization. Research thrust is needed to apply synthetic seed technology for production of secondary metabolites to ease out collection pressure on natural habitats. Emphasis should also be directed to exploit the technology for reintroduction of plants to natural habitat.

Regarding conservation meager but encouraging work has been done so far. The challenge lies in optimizing repeatable working cryopreservation protocol for application in germplasm conservation in the genebanks. Monitoring of genetic stability of thus propagated and conserved medicinal plants to ensure conservation for sustainable utilization is challenging but an important aspect requiring focused research efforts. Encapsulation of transformed hairy roots having potential industrial significance is one of the thrust areas which need attention.

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Cash Crops: Synseed Production, Propagation, and Conservation

Zishan Ahmad and Anwar Shahzad

Abstract Advancement in encapsulation technique has provided excellent opportunity for the improvement of crops, trees, and several other plant species. The application of encapsulation technology in the field of agriculture opens new vistas for plant propagation, conservation, and delivery of germplasm. The synthetic seed was a promising application in the propagation of vegetatively propagated and polyploidy species that are hard to propagate. In majority of the cash crop, propagation through seed is unsuccessful due to heterozygosity, low germination of seed, and absence of normal endosperm. Considering these problems, a great interest has been developed to use encapsulation techniques for the propagation, conservation, and accelerated germplasm exchange. The proposed study deals with a piece of up-to-date information on the synseed development in various cash crops.

Keywords Cash crop · Encapsulation · Germplasm exchange

1 Introduction

A cash crop or profit crop is an agricultural crop which is grown to sell for profit and considered as a precise method that has validated to raise affordable food in extravagant quantities. It can be described as a farm product that is sold on a commercial agricultural market and also called as commercial farming or cash cropping. It is significantly associated with the importance of food security at the level of governments and farm household in agriculture sector of the emerging countries. A cash crop brings consequential emolument and employment opportunities to the rural economy and promotes economic diversification. From several years, cash crops bring impetus to agricultural innovation by raising capital for farm investment and earning revenue for the government. The production of cash crop

Z. Ahmad · A. Shahzad (✉)
Plant Biotechnology Section, Department of Botany, Aligarh Muslim University, Aligarh, Uttar Pradesh, India
e-mail: ashahzad.bt@amu.ac.in

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empowers farmers and farm workers to advance their living standard, thus contributing to food security. In the beginning, cash crop farming was a small part of a farm's total yield, while nowadays, approximately all the crops are mainly grown for profit particularly in developed countries, while in the case of underdeveloped countries, it is expanded to attract demands from the developed countries to enhance the export value.

Like any farming exercise, cash crop farming also required management against various types of risk. The communities with a high dependency on cash crop farming will face a drop in incomes due to lack in proper management. Different biotic and abiotic factors are the stationary factor that restricts the production of cash crop plants. In the majority of the cash crop, propagation through seeds found to be abortive. The reason might be the seeds heterozygosity, tiny size, and low germination and presence of reduced endosperm (Saiprasad 2001). The presence of seedless varieties is also one of the restrictions for propagation (Saiprasad 2001). The propagation through a conventional method like grafting, air layering, stooling, etc. to ameliorate cash crop exists but is restricted due to unusual juvenility that has made these techniques wasteful of time and bunglesome (Litz and Jaiswal 1991).

Plant tissue culture (PTC) technology found to be effective against these problems associated with the propagation of cash crops. A potent regeneration protocol is prerequisite for the advancement of cash crops through different biotechnological strategies. In most of the cases, the characteristics of the cash crops restrict their conservation through traditional methods due to several reasons such as desiccation-sensitive intermediate or recalcitrant seed and long-duration storage problems. A high rate of occurrence of fungi and their infection are altogether responsible for low storage period (Rai et al. 2009). Moreover, collection of germplasm from field gene banks is more prone to exposure to the natural apocalypse and pathogens attacks (Rai et al. 2009). According to Chaudhury and Malik (2003), the vegetative nature of material restricts the allocation of genetic resource from gene bank, and the action will also promote disease transfer. To evade these problems, an accelerated approach has been established for the application of synseed technology for the preservation and delivery of germplasm in cash crops. The present communication deals with the up-to-date information on the application of synseed technology in various cash crops for their conservation and germplasm exchange.

2 Synseed: Background, Procedure, and Advantage

Murashige (1977) first gives the concept of synthetic seed; however, Kitto and Janick (1982) published their first work on synseed approach. They use polyoxythylene glycol as a coating mixture to encapsulate the carrot somatic embryo to obtain desiccated synthetic seed. However, the first encapsulation using alginate hydrogel was achieved by Redenbaugh et al. (1984), for the encapsulation of SE in alfalfa. Since then various studies have been conducted so far on several plant species such as cereals, fruits, vegetables, ornamentals, medicinal plants, forest

trees, and orchids (Bapat et al. 1987; Corrie and Tandon 1993; Rout et al. 2001; Naik and Chand 2006; Micheli et al. 2007; Faisal and Anis 2007; Gantait et al. 2015; Rathore and Kheni 2017; Prakash et al. 2018; Khan et al. 2018; Kundu et al. 2018).

In the beginning, the synthetic seed technology was used to encapsulate generally somatic embryos with an objective to be used for long-distance transport, short- and long-term storage, but later, the techniques were also found to be useful for the encapsulation of vegetative propagules, viz., apical shoot buds, axillary buds, NS, etc. which have been seen as optional to SE (Ara et al. 2000; Bapat and Mhatre 2005; Rai et al. 2008; Gantait et al. 2015; Rathore and Kheni 2017; Prakash et al. 2018). Encapsulation of above propagules was found to be more advantageous for those crops plants, where somatic embryo could not substantiate. In such problems, synthetic seed technology is found to be more applicative for propagation and transport of germplasm (Rao et al. 1998). Two approaches could be tried for the synseed production (1) hydrated and (2) desiccated. Hydrated synthetic seeds could be obtained by encapsulation of propagules in hydrogel (Redenbaugh and Walker 1990). Different coating agents are being used for the encapsulation such as sodium alginate (SA), potassium alginate, carrageenan, SA with gelatin, sodium pectate, carboxymethyl cellulose, etc. However, among these coating agents, SA was used frequently (Redenbaugh et al. 1987; Rao et al. 1998; Ara et al. 2000; Faisal and Anis 2007; Shaheen and Shahzad 2015).

Direct sowing of synthetic seeds ex vitro gives economically meaningful and worthwhile approaches for the artificial seeds to plantlet recovery. Although various plants are micropropagated through PTC, however germplasm exchange is restricted, and hence synthetic seed was found to be an ideal delivery system with easy handling and transport (Mandal et al. 2000). However, the conversion of synseed into plantlets is limited to only a few plant species; the reason suggested is low nutritional availability, which would be overcome by enriching the gel matrix with hormones or by adding them into the planting media (Singh et al. 2006, Faisal and Anis 2007; Sharma et al. 2013). Application of nutrient source, growth regulators, antibiotics, fungicides, etc. in the gel matrix was found useful for those species where nutrient availability is low due to the absence of endosperm in natural seed, and conversion ability was also found to be improved (Redenbaugh et al. 1987; Bapat and Mhatre 2005). The appearance of root and shoot from the propagules was sometimes found to be hindered due to gel capsule; thus self-breaking alginate gel bead approaches were found to be effective to overcome this shortcoming. The lack of natural endosperm makes synseed challenging to root and shoot emergence from the propagules (Onishi et al. 1994).

Onishi et al. (1994) found a smooth emergence of shoot and root from the beads pretreated with KnO_3. Various workers have also achieved similar kind of results in different plant species (Guerra et al. 2001; Arun Kumar et al. 2005). To obtain the hydrated seeds, the propagules such as SE, shoot bud, NS, etc. were isolated from in vitro culture followed by mixing with encapsulation matrix composed of SA (0.5–5.0% w/v) dropped in $CaCl_2$ solution (30–150 mM) as complexing agent and which took approximately 30–40 min to harden in to the beads. The hardness is due to supplant of Na^+ by Ca^+ leading to the formation of calcium alginate (Redenbaugh

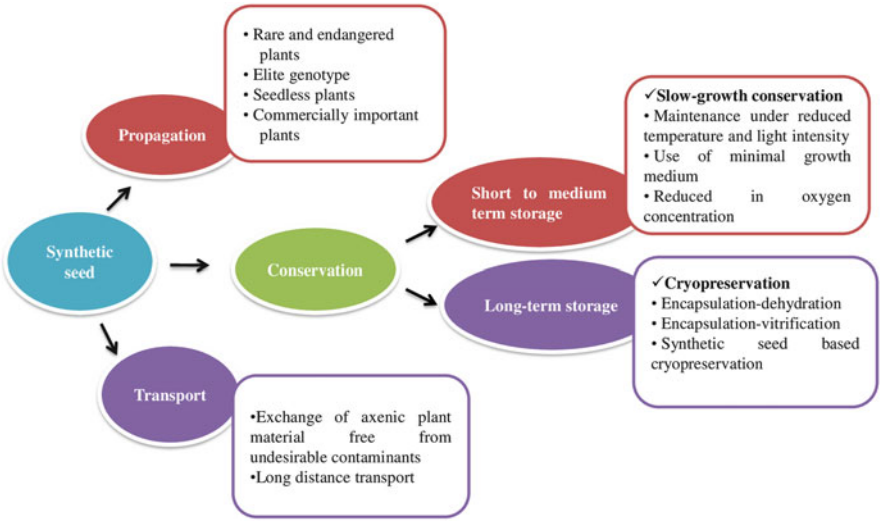

Fig. 1 Advantages of synthetic seed

and Walker 1990; Ara et al. 2000). The hardening process is directly affected by SA and $CaCl_2$ dosage. However, the concentration also depends on type of explants used; such kind of beads was washed with DDW and cultured on various nutrient media or other substrates like wet filter paper, cotton paper, or soilrite to obtain the plantlets.

3 Advantage of Synthetic Seed

The approach of synseed divulges a new perspective in the area of plant biotechnology. Synthetic seed can be useful in various ways for the utilization of germplasm. Thus the primary aim in synseed technology was to deal with SE in such a way that mimicked zygotic embryo during storage and other application. The potential use of synthetic seed has been shown in Fig. 1. It is one of the excellent techniques for the propagation of rare and endangered plants, elite genotype and rare hybrid plants, etc. (Mandal et al. 2000). The production for hybrid seed like in cash crops or other traditional propagation techniques is too high, and hence synseeds become low-cost alternatives. Synthetic seed could be effectively planted either on semisolid medium or in a planting substrate such as vermicompost, soilrite, etc. to achieve complete plantlet formation (Mandal et al. 2000). For those species which must sustain their seed once in a year, synthetic seeds overcome the problems as it is available throughout the year. The conversion of beads into complete plantlets in in vitro conditions involves pathogen-free and controlled environment for short- to medium- or long-term storage duration (Engleman 2003). And it can be achieved by

alteration in the environmental conditions and/or in nutrient medium composition such as prolongation under low temperature or light intensity, application of growth inhibitor such as ABA (abscisic acid), reduction in oxygen concentration, and use of osmoticum (Gupta and Mandal 2003).

4 Synthetic Seed Production in Some Important Cash Crop

Several reports are available on the development of synthetic seeds for propagation and conservation of cash crops (Table 1).

4.1 Cassava (Manihot esculenta Crantz, Family: Euphorbiaceae)

Cassava is considered to be an essential source of energy in tropical countries. In India, it is widely used for food security and also in the dye industry to its capability to survive and propagate in marginal and wasteland in comparison to other crops. Generally, stem cuttings were carried out for the commercial propagation, however, low rate of multiplication and difficulty in transporting of plant material, this crop is unable to draw much attention in the international market. Synthetic seed production in cassava emerged as a tool to deal with these problems effectively. Hegde et al. (2016) developed a protocol for the encapsulation of NS of cassava variety (H-226) with a varying concentration of SA, viz., 2, 3, and 4% coupled with MS salts without calcium salts and treated with varying level of 75 mM, 100 mM, and 125 mM calcium chloride solution ($CaCl_2.2H_2O$) as a complexing agent. Among the different concentration of SA and calcium chloride, a combination of 3% SA and 100 mM $CaCl_2$ was found useful in terms of ideal beads formation, and a frequency of 96.67% regeneration was achieved in in vitro condition. However higher doses of SA (4%) and $CaCl_2$ (125 mM) exhibited low germination frequency. Various storage periods were analyzed also, and germination % decreased as the days advanced. A maximum of 93.33% germination was observed at 0 day, while no germination was observed after 35 days of storage period. The optimized ratio of SA and $CaCl_2$ along with MS can surely be utilized as a promising alternative to the propagation of cassava.

4.2 Cotton (Gossypium hirsutum L., Family: Malvaceae)

Cotton being a significant source of textile fiber in worldwide often faces technical challenges to producing sterile lines due to cross-pollination. It is hard to maintain

Table 1 Synthetic seed production in some cash crop species

Plant species	Conservation method	Plant material	Reference
Ananas comosus (pineapple)	Low-temp. storage	SBs	Soneji et al. (2002), Gangopadhyay et al. (2005)
	Encapsulation-dehydration	ST	Gonzalez-Arnao et al. (1998)
	Encapsulation-vitrification	SA	Gamez-Pastrana et al. (2004)
Citrus sp.	Low-temp. storage	SEs	Antonietta et al. (2007)
	Encapsulation-dehydration	SEs	Gonzalez-Arnao et al. (1998, 2003)
	Low-temp. storage	SEs	Singh et al. (2007)
Cannabis sativa	Low-temp. storage	SBs	Lata et al. (2009)
Gossypium hirsutum (cotton)	Low-temp. storage	SBs	Hu et al. (2015)
Hordeum vulgare (barley)	Low-temp. storage	MdE	Datta and Potrykus (1989)
Ipomoea batatas (sweet potato)	Encapsulation-dehydration	SA	Doussoh et al. (2018)
Manihot esculenta (cassava)	Low-temp. storage	NS	Hegde et al. (2016)
Mangifera indica (mango)	Encapsulation-dehydration	SEs	Wu et al. (2003)
Morus indica (mulberry)	Low-temp. storage	ABs	Bapat et al. (1987), Pattnaik et al. (1995)
	Encapsulation-dehydration	ST	Niino and Sakai (1992), Niino et al. (1992)
Oryza sativa (rice)	Low-temp. storage	SEs	Kumar et al. (2005)
Vitis vinifera (grape)	Encapsulation-dehydration	ST	Plessis et al. (1993)
	Encapsulation-dehydration	ABs	Zhao et al. (2001)
	Encapsulation-dehydration, encapsulation-vitrification	ECS	Wang et al. (2002, 2004)
Zea mays var. saccharata (sweet corn)	Low-temp. storage	SEs	Thobunluepop et al. (2009)

ABs axillary buds, *ECS* embryonic cell suspension, *MdE* microspore-derived embryo, *SBs* shoot buds, *SEs* somatic embryo, *ST* shoot tips

the lines or F_1 hybrid varieties of cotton with extended period of flowering. The propagation of cotton with plant tissue culture and synthetic seed is an alternative approach to maintain the F_1 hybrid. Hu et al. (2015) established an encapsulation protocol of axillary buds of *Gossypium hirsutum*. 3% SA in MS medium (liquid) with 100 mM $CaCl_2$ was found useful for ideal beads formation. The medium comprised of MS + 0.5 mg L^{-1} IBA + 0.5 mg L^{-1} NAA was found a suitable treatment for the maximum regeneration, where 95% synthetic seeds germinated; however, rooting was observed in 35.8% of synthetic seed only with an average

length 2.2 cm after 30 days of inoculation. In another experiment, they used a combination of 3% SA + 1% chitosan dissolved in 85 mM acetic acid and solidified liquid containing 200 mM $CaCl_2$ + 0.02% NaOH, wherein 96.1% synthetic seed germination was observed on the same treatment. Among the different storage temperature (4–20 °C) and storage duration (0–4 week), a temperature of 20 °C was prominent at 0 week of storage duration wherein a maximum of 95.2% germination was recorded. Moreover, germination was decreased as the storage duration increased. The above protocol can be a promising tool in agriculture for cotton to maintain the F_1 hybrid.

4.3 Rice (Oryza sativa L., Family: Poaceae)

Kumar et al. (2005) encapsulated somatic embryos of hybrid rice in SA complex prepared in the nutrient solution of MS, PGR, and protectants for the artificial endosperm to increase the germination and transformation ability. The obtained synseed encapsulated in the matrix supplied with MS + IAA 0.5 mg L^{-1} + NAA0.5 mg L^{-1} + BA 0.5 mg $L^{-1}$and charcoal (1.25%) was found to be effective, and germination frequency of 30% with 27% conversion efficiency was observed. They further used bavistin and streptomycin in matrix; however, no significant effect was observed. Moreover, application of self-breaking gel bead technique enhances the germination and conversion frequency of 52 and 47% of synthetic seeds.

4.4 Banana (Musa sp., Family: Musaceae)

Banana is one of the major cash crops in the world. The vegetative propagation through suckers naturally occurs; however due to a lesser number of sucker production, their propagation at massive scale is limited. PTC technology brings an advantage to meet the world demands of banana. Micropropagation through shoot tip explants has been reported well (Ganapathi et al. 1992, 2001; Suprasanna et al. 2001). However, a remarkable effort has also been carried out for the propagation and conservation of banana using encapsulation technology. Banana cv. Basrai was used as a source of explants and encapsulated in 3% SA supplemented with various gel matrices (Ganapathi et al. 1992). Application of White (1939) medium was found useful for the maximum conversion of encapsulated ST into plantlets. Further, the application of 0.1% activated charcoal and a mixture of antibiotics to the gel matrix overcomes the problem of bacterial infection. The transport of germplasm of banana using encapsulated ST is quite affordable, less expensive, and safer than sucker's transportation. The encapsulation of banana SE was first reported by Ganapathi et al. (2001). In their findings, they recorded that the constituents of gel matrices and substrate effected the conversion of synseed into plantlets. However,

5% SA was the most promising concentration of gel matrix for the encapsulation and maximized conversion after incubation on to MS medium. There are various reports available for the in vitro conversion of banana cultivar using minimal growth medium. For long-term storage of banana cultivars (Banerjee and De Langhe 1985), cryopreservation was successfully achieved (Abdelnour-Esquivel et al. 1992; Panis et al. 1990, 1996; Cote et al. 2002).

4.5 *Sweet Potato (Ipomoea batatas Lam., Family: Convolvulaceae)*

Ex situ conservation of vegetatively propagated species such as sweet potato is one of the difficult tasks. Doussoh et al. (2018) established a protocol for the conservation of sweet potatoes, their genetic resources, and long-distance transport using synthetic seed technology. They achieved short-term preservation by developing the encapsulation-dehydration technique. Two varieties of Koïdokpon and Dokoui carotte were used for growing shoot collections and disinfected with 10% sodium hypochlorite. The evaporative dehydration was carried out using silica gel during 5 and 6 h before encapsulation. The encapsulation was carried out by using 3% SA + 1.30 CaCl followed by conservation in Eppendorf tubes at 20 °C in batches for 15 days and 90 days. After that, inoculated on a nutrient medium consisting of 0.15 mg/L BAP, 0.2 mg/L NAA, 0.08 mg/L GA3, and 80 mg/L adenine sulfate followed by evaluation of survival and regeneration rate. Highest survival rates of 59.26% and 37.04% and highest regeneration rates of 37.04% and 11.11% were recorded, respectively, with the landraces "Koïdokpon" and "Dokoui carotte" when the apices were dehydrated for 6 h and then stored for 15 days. However, as the storage duration increased, the regeneration rate decreased accordingly. Similarly, a significant difference was also observed between the landraces regarding their preservation. The developed protocol can surely be used for the conservation of endangered sweet potato.

4.6 *Grapes (Vitis spp., Family: Vitaceae)*

Grapes are widely used cash crop grown for profit. Grapes are heterozygous with inbreeding depression, and hence it is hard to produce individual genotype by seed (Gray and Meredith 1992). The advantage of encapsulation technology overcomes these problems, and enhanced rate of propagation and conservation can be achieved. Das et al. (2006) first encapsulated the somatic embryo of *Vitis vinifera* obtained from leaf explants. The encapsulated somatic embryos were in 2% alginate and plated on 0.7% agarified medium composed of B_5 macrosalts (half strength), MS macrosalts (full strength), 3% sucrose, and 2.9 μM gibberellic acid. B_5 medium

supplemented with 0.04 μM ABA was used for transferring the embryos for 4–6 weeks before encapsulation resulted in the enhanced storage of up to 90 days without any declined in the conversion efficiency. The cryopreservation methods have also been used for the successful conservation by the various workers (Plessis et al. 1993; Zhao et al. 2001; Wang et al. 2002, 2004).

4.7 *Pomegranate (Punica granatum L., Family: Lythraceae)*

Pomegranate is one of the pharmacologically important cash crops of tropics. Naik and Chand (2006) developed protocol for the encapsulation of axenic NS in calcium alginate hydrogel containing MS + BA 4.44 mM + 0.54 mM NAA. Among the different concentrations of SA (1–6%) and calcium chloride (50–125 mM), a complex of 3% SA and 100 mM calcium chloride was found optimum for the formation of ideal beads. Similarly, among the different planting media tried, a combination of MS + BA 4.44 mM + 0.54 mM NAA was witnessed with the highest regrowth in the shoot. The obtained encapsulated NS could be stored at 4 °C for 30 days.

4.8 *Litchi (Litchi chinensis Sonn., Family: Sapindaceae)*

Litchi is a valuable crop across the world because of its use in the wine industry. Most of the cultivars are propagated through air layering and marcottage methods. However, the storage duration was a limiting factor for successful conservation and long-distance transport, and hence the encapsulation technology was found to be an appropriate technique to overcome the problems. Das et al. (2016) used cotyledonary stage somatic embryo developed from a zygotic embryo for the encapsulation using 2% alginate gel. Nitsch and Nitsch basal medium augmented with 0.7% agar, 3% sucrose, and 1 mg L^{-1} GA was used as nutrient matrix. The germination frequency on agar medium was higher (44.3%) for encapsulated somatic embryos than non-encapsulated embryos (23.7%). Similarly, the effect of ABA (abscisic acid) was also studied on agar medium for encapsulated somatic embryos wherein ABA at 0.02 μM was found optimum and 69.1% germination was observed. The obtained plantlets were further transferred to the field soil. However, further effort is required to apply the applications of encapsulation technology in litchi.

4.9 *Barley (Hordeum vulgare L., Family: Poaceae)*

The microspore-derived embryo of barley has been used for encapsulation (Datta and Potrykus 1989). A combination of SA and $CaCl_2$ was used for encapsulation and

to achieve the artificial beads. Maximum regeneration was observed in the encapsulated embryo rather than non-encapsulated embryos. Moreover, the germination capacity persisted up to 6 months for encapsulated embryo, while non-encapsulated embryos were unable to survive for more than 2 weeks. The obtained artificial seed can be surely used for delivery of germplasm in barley.

4.10 Sweet Corn (Zea mays L. var. saccharata*, Family: Poaceae)*

Several reports are dealing with somatic embryogenesis in sweet corn; however, some limitations such as low storage duration and viability of seed were observed. A protocol has been developed for somatic embryo production coupled with encapsulation technology by Thobunluepop et al. (2009) in sweet corn. They used immature embryoids obtained from callus, inoculated them on medium comprised of N6 + 2, 4- D 2 mg L^{-1} + Sucrose 60 g/L. An application of 3% (w/v) SA with 100 mM $CaCl_2$ was found suitable as encapsulation matrix. Synseed treated with 60 gl^{-1} sucrose was found with enhancing survival rate of 44% after 8 days of germination test when stored at 15 ± 2 °C for 2 weeks wherein 91% of which were normal seedling and 9% were abnormal seedling. The obtained results witnessed the scope of synseed production; however, more researches are required for the perfection of technology.

4.11 Cannabis (Cannabis sativa L., Family: Cannabaceae)

Cannabis is used as black market cash crop, well known for its medicinal and therapeutic potential. The allogamous nature of this species limits the potency and efficacy of propagated through the natural seed. Synthetic seed technology has been developed for in vitro propagation of *C. sativa* (MX-1), using encapsulation techniques (Lata et al. 2009). Application of 5% SA with 50 mM $CaCl_2$ was found as best gel matrix and used to encapsulate the axillary buds. The encapsulated beads were inoculated on MS + TDZ 0.5 mM and PPM (0.075%), where they exhibit best regrowth and conversion frequency in in vitro condition. However, under in vivo condition, 100% conversion of encapsulated explants was achieved on 1:1 potting mix—fertilome—with coco natural medium, moistened with full-strength MS medium without TDZ, supplemented with 3% sucrose and 0.5% PPM. The obtained plantlets were acclimatized and successfully transferred to the soil conditions.

4.12 Citrus (Citrus reticulata, Family: Rutaceae)

Citrus is grown at a greater extent in tropical and subtropical regions in the world. A high quality of citric acid and vitamin C is present in juice obtained from citrus. Antonietta et al. (1999) first tried to encapsulate the somatic embryos of *Citrus reticulata* Blanco through alginate encapsulation. Different combinations for artificial endosperm have been tried such as encapsulation with growth regulator free or augmented with GA_3. However, artificial endosperm containing GA_3 has exhibited maximum potential in plantlet conversion on agar gel medium and was also found useful in short-term storage of ST at 4 °C for 30 days. Whereas application of germicide PPM and fungicide (Thiophanate-methyl) on the performance of encapsulated embryos during the various storage period were evaluated by Antonietta et al. 2007. A maximum of 60 days of storage periods was found suitable at 4 °C without a major decline in viability. There are various reports available also dealing the in vitro conservation of citrus species (Gonzalez-Arnao et al. 1998, 2003; Malik and Chaudhury 2006).

5 Conclusion and Future Prospects

A remarkable advancement has been carried out in in vitro propagation via synthetic seed in various cash crops. It offers excellent scope for the conservation and germplasm exchange of different cash crops. However, direct sowing of synseed is a limitation due to low survival (Jung et al. 2004). A viable micropropagule production of large scale in a cost-effective manner is a first demand for practical application of synseed (Ara et al. 2000). Therefore purification in the protocol is prerequisite to achieving suitable beads complexing to advance the propagation techniques through synseeds. The encapsulation technique is a high-grade approach for the conservation and delivery of those which have direct seed propagation limitation and best germplasm and plants which do not produce seed. However, a concerted efforts are required for the development of synseeds suitable to conversion and plant growth in soil conditions.

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Synthetic Seeds of Two Aquatic Plants

Maurizio Micheli and Carla Benelli

Abstract Aquatic ornamentals are generally used for aquarium decorations because of their colored foliage and attractive appearance. *Cryptocoryne* species are popular due to their easy growth and capacity to thrive for a long time. *Cryptocoryne lutea* is an excellent mid-ground plant with bright green or bronzy oblong leaves. *Rotala rotundifolia* is characterized by a rapid growing stem and beautiful growth pattern; for these features it is considered the ideal aquarium plant for beginners. These aquatic plants are usually easily propagated by cutting, but previous studies allowed to develop a suitable protocol of micropropagation. A renewed interest is due to the possibility to obtain synthetic seeds by in vitro-derived propagules to be used in aseptic or in ex vitro conditions for propagation system.

Keywords Artificial seed · Calcium alginate · Conversion · Non-embryogenic propagule · Microcutting · Plant tissue culture

1 Introduction

Aquatic plants (or hydrophytes) are plants that have adapted to live in or on aquatic environments. Great parts of them are represented by ornamental species, and they are cultured for their beauty, but also to maintain water quality in aquarium. Their selection is based on shape, leaf color, and size (Stodola 1980). The demand is still limited to specific markets, but the production of aquatic plants has been developing rapidly, and the industry requires a continuous supply of high-quality plants on a large scale. *Cryptocoryne* and *Rotala* gen. are included into the list of the most common aquatic plants: native in vast areas of Southeast Asia and Indonesia, they

M. Micheli (✉)
Department of Agricultural, Food and Environmental Sciences (DSA3), University of Perugia, Perugia, Italy
e-mail: maurizio.micheli@unipg.it

C. Benelli
National Research Council - BioEconomic Institute (CNR-IBE), Sesto Fiorentino, Firenze, Italy

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are submerged or emerged (Kasselmann 1999). Propagation by seed and division of rhizomes are not productive systems and require a long time. Consequently, these methods are not used (Windelow 1987). Most plants commercialized in the USA are collected in their natural habitat. The clumps are divided into single plants, acclimatized and grown in baths before sale (Micheli et al. 2006). The introduction of tissue culture to produce these valuable aquatic plants holds several advantages for industry. It can provide good-quality plants without pest or disease at a competitive price for the export market; in addition, large-scale plant production can be programmed according to export requirements (Yapabandara and Ranasinghe 2006). But the information about in vitro propagation of aquatic plants is limited and fragmentary, perhaps because the experimental activities are conducted within private companies (Kane et al. 1988, 1990; Staritski 1977). Nevertheless, previous studies showed that *Cryptocoryne lutea* and *Rotala rotundifolia* can be easily proliferated in in vitro conditions (Micheli et al. 2006). In addition, good levels of multiplication can be accompanied by satisfactory roots formation: as consequence, it is possible to avoid a separate stage dedicated to rooting, but to transfer the plantlets directly to the aquarium for acclimatation after the proliferation phase (Micheli et al. 2006). The research is investigating to find new propagation procedures able to join the advantages of micropropagation (high productive efficiency, sanitary plant conditions, and reduced space requirements) with the technologic characteristics of the zygotic seed, as handling, storability, and transportability (Micheli et al. 2003), represented by the synthetic seed technology. Murashige (1978) gave the first definition of synthetic seed as an encapsulated single somatic embryo (SE) inside a covering matrix of calcium alginate. The encapsulation was proposed to safeguard the SEs from mechanical damage during handling in the nursery and transportation in the farm, as well as to provide nutrients (artificial endosperm) during their natural evolution in plantlets (conversion) (Micheli and Standardi 2016). Recently, different explants were tested to produce synthetic seeds, as nodal segments (microcuttings). In these explants the absence of root primordia is coupled with their inability to form adventitious roots spontaneously (Standardi and Micheli 2013); nevertheless, in *Cryptocoryne* and *Rotala* gen., characterized by a high rooting ability, encapsulated microcuttings are able to convert naturally in whole plantlet when they are sown in suitable conditions. The encapsulation offers an efficient and cost-effective system for clonal propagation of plant species and could be used to produce synthetic seeds for restoration purposes and for the exchange of axenic plant material between laboratories (Fontanili et al. 2015).

2 Previous Experimental Experiences

Cryptocoryne lutea and *Rotala rotundifolia* were previously studied in order to individuate a suitable protocol for micropropagation (Micheli et al. 2006). The shoot proliferation was obtained in Linsmaier and Skoog (LS) medium (Linsmaier and Skoog 1965) enriched with 0.5 mg l^{-1} naphthalene acetic acid (NAA) and two

concentrations of 6-benzylaminopurine (BAP) (1 and 4 mg l^{-1}) (Note 1). In all species, the medium containing 4 mg l^{-1} of BAP resulted in higher multiplication rates (Micheli et al. 2006). The color of the proliferated shoots was always light green in *Cryptocoryne lutea* and dark green in *Rotala rotundifolia*, proper and typical colors of the two species. In all cases the proliferation was accompanied by root formation. The lower concentration of BAP (1 mg l^{-1}) induced a higher number and length of roots (Micheli et al. 2006). In *Cryptocoryne lutea* the roots appeared fleshy, while in *Rotala rotundifolia* they were fibrous. In general, considering also the results obtained during the acclimation, with two substrates (natural and commercial substrates), the conclusion was that the application of 4 mg l^{-1} of BAP was more productive because it induced a higher number of shoots, while the substrate poor in cytokinins could be employed to obtain a better quality of rooting (Micheli et al. 2006).

The high rooting capacity of shoots of *Cryptocoryne lutea* and *Rotala rotundifolia* induced to assume that nodal microcuttings could be able to develop a whole plantlet (conversion) without inductive treatments and can be employed to produce synthetic seeds of *Cryptocoryne lutea* and *Rotala rotundifolia*. In fact, subsequent studies confirmed that the synthetic seeds, obtained from the encapsulation of microcutting showed 100% of conversion in both species after sowing in aseptic conditions (data not published).

3 Encapsulation

The protocol to obtain synthetic seeds of *Cryptocoryne lutea* and *Rotala rotundifolia* is following described.

3.1 Laboratory Facilities

Graduate glasses, pipettes, pipettor, magnetic stirrer, spin bar, analytical balance, pH meter, NaOH and HCl solutions (0.1 N), glass jars, autoclave, horizontal flow cabinet, forceps, scalpels, blades, electric incinerator, and growth chamber.

3.2 Plant Material

In aseptic conditions, microcuttings (nodal segments of 3–4 mm) of *Cryptocoryne lutea* and *Rotala rotundifolia* are excised by shoots proliferated, maintained in glass jars on agarized LS medium (Linsmaier and Skoog 1965) enriched with 0.5 mg l^{-1} NAA and 1 mg l^{-1} BAP (Note 1) in growth chamber (Note 7).

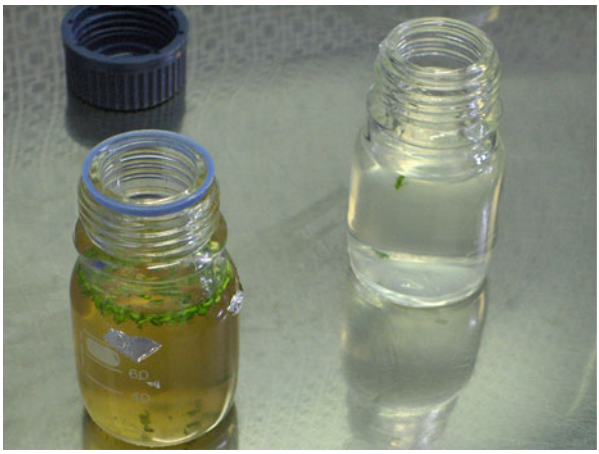

Fig. 1 Coating and complexing solutions (*from left to right*) employed to obtain *Cryptocoryne lutea* synthetic seeds

3.3 Encapsulation Solutions

Coating, complexing, and rinsing solutions are essential to carry out the encapsulation of microcuttings (Fig. 1).

Their basal components are:

1. Artificial endosperm. It is composed by half strength LS basal medium enriched with 0.02 mg l^{-1} NAA, 0.5 mg l^{-1} BAP, and 50 g l^{-1} sucrose (pH 5.7) (Note 1).
2. Coating agent. It is represented by alginate sodium salt with medium viscosity.
3. Complexing agent. It is represented by calcium chloride anhydrous.

The artificial endosperm is the common constituent of each solution.

Coating (2.5% w/v) and complexing (1.1% w/v) agents are separately dissolved into artificial endosperm to obtain, respectively, coating and complexing solution (Note 1).

The rinsing solution is composed only by the artificial endosperm (Note 1).

3.4 Encapsulation Procedure

(a) In aseptic conditions, each microcutting is immersed in the coating solution for a few seconds (Note 2).
(b) Each drop, containing one microcutting, is then immerged into the complexing solution for 25–30 min (Note 3) to obtain beads (Note 4).
(c) All beads are washed at the same time in the rinsing solution for 10–15 min, 2–3 times (Note 5).

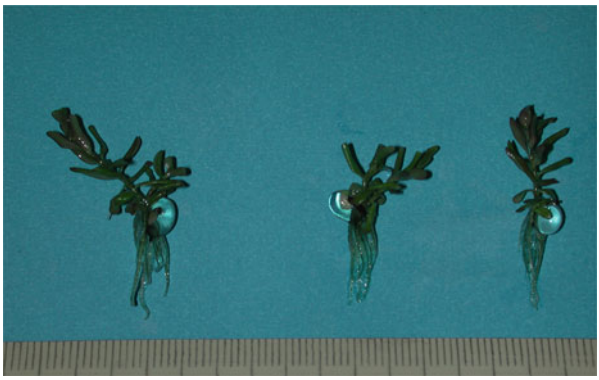

Fig. 2 Conversion of synthetic seeds of *Rotala rotundifolia*

3.5 Sowing Medium

Full-strength LS basal medium enriched with 30 g l^{-1} sucrose and 7 g l^{-1} agar (pH 5.7) (Note 1).

3.6 Sowing Procedure

(a) After rinsing the encapsulated microcuttings (synthetic seeds) are aseptically transferred into closed glass jars (500 ml), each containing 100 ml of sowing medium and 8–10 synthetic seeds (Note 6).
(b) Cultures are transferred into the growth chamber (Note 7).
(c) Viability, regrowth, and conversion of encapsulated microcuttings of *Cryptocoryne lutea* and *Rotala rotundifolia* (Fig. 2) are evaluated after 30 days (Note 8).

3.7 Future Perspectives

Previous study on *Cryptocoryne lutea* and *Rotala rotundifolia* micropropagation was completed by the acclimatation phase directly into the aquarium. It was easily achieved by transferring the plantlets on a soil composed of peat, fine clay, and sand (1:1:10/v:v:v) or on COMPO CACTEA®, a commercial substrate used for the cultivation of *Cactaceae* (Micheli et al. 2006). This result indicates the possibility to use also synthetic seeds of two species in ex vitro conditions, inducing conversion directly into the aquarium. They could be proposed in the future as commercial biotechnological products, able to assure the development of high-quality plantlets with regard to the genetic and sanitary aspects.

4 Notes

1. All containers, solutions, and media are autoclaved at 115 °C for 20 min just after their employment.
2. Sodium alginate is frequently used due to its moderate viscosity, low spin ability of solution, low toxicity, quick gelation, low cost, and good biocompatibility. As an alternative, different substances were proposed, like mixture of sodium alginate with gelatin, potassium alginate, polyco 2133, carboxymethyl cellulose, carrageenan, gelrite, guar gum, sodium pectate, and tragacanth gum (Rai et al. 2009; Redenbaugh et al. 1993; Saiprasad 2001).
3. During the complexation, step ion exchange occurs through the replacement of Na^+ by Ca^{++} forming calcium alginate (Ara et al. 2000; Redenbaugh and Walker 1990) by ionic cross-linking among the carboxylic acid groups, and the polysaccharide molecules form a polymeric structure called "egg-box" (Barbotin et al. 1993). Hardening of bead is affected by the concentration of sodium alginate and calcium chloride, and it may vary also by the complexation time (Standardi and Micheli 2013; Benelli et al. 2017).
4. Each encapsulated portion of in vitro-derived plant tissue possessing the ability to evolve in shoot (not into a whole plantlet) can be defined as *bead* (Benelli et al. 2017) or *capsule* (Standardi and Micheli 2013). Beads can be useful for the exchange of germplasm of elite genotypes and axenic plant material between laboratories due to the small size and relative ease of handling (Benelli et al. 2017).
5. The final washing is essential to remove the toxic residual ions of chloride and sodium.
6. Synthetic seeds are sown inserting them just for some millimeters into the substrate, in order to avoid asphyxia of living tissues, respecting the propagule growth polarity.
7. Growth chamber conditions: 21 ± 2 °C temperature, 40 μmol m^{-2} s^{-1} photosynthetic photon flux density, and 16 light hours photoperiod.
8. *Viability*: green appearance of encapsulated propagule, with no necrosis or yellowing, after sowing (Micheli and Standardi 2016). *Regrowth*: capacity of a bead to develop at least a new shoot after sowing (Benelli et al. 2017). The term regrowth is more appropriate than "germination" used by many other authors, in spite of the absence of direct root regeneration (Benelli et al. 2017). *Conversion*: ability of a synthetic seed to evolve in a whole plantlet after sowing (Micheli and Standardi 2016).

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Synthetic Seed Technology in Forest Trees: A Promising Technology for Conservation and Germplasm Exchange

Suprabuddha Kundu, Monoj Sutradhar, and Umme Salma

Abstract Forest trees are less domesticated in comparison with the agricultural plants, and even the seed produced during breeding programs is genetically diverse. The increasing global need for food and fibre results in new demands for the efficiency of wood production. The exploitation of forest area emphasizes the importance of the immediate development of conservation strategies for forest tree species. Synthetic seed technology is an advanced and highly increasing forte of plant biotechnological research. For the last two decades, intensive research efforts have been made on synthetic seed production in a number of plant species. The technique involves the use of any meristematic tissue like shoot tip, nodal segment or somatic embryo for large-scale propagation and germplasm exchange between laboratories, thus lowering the dependence on micropropagation and minimizing its relevant expenditures. In most of forest species, seed propagation has not been successful because of heterozygosity of seeds, minute seed size, presence of reduced endosperm and low germination rate. Many species have desiccation-sensitive intermediate or recalcitrant seeds and can be stored for only a few weeks or months. Under these circumstances, increasing interest has been shown recently to use encapsulation technology for propagation and conservation. The technology also provides its importance in ex vitro conservation as the encapsulation protects the plant sample from the unfavourable effects of toxic cryoprotectants and post-storage damages. After an introduction on the main procedures for synseed preparation, this chapter provides information on the protocols that have been developed for the encapsulation of various explants from forest tree species.

Keywords Calcium chloride · Conservation · Encapsulation · Forest tress · Sodium alginate · Synthetic seed

S. Kundu (✉) · M. Sutradhar · U. Salma
Department of Agricultural Biotechnology, Faculty of Agriculture, Bidhan Chandra Krishi Viswavidyalaya, Mohanpur, Nadia, West Bengal, India

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Abbreviations

ABA	Abscisic acid
BA	N_6-benzyladenine
CC	Calcium chloride
IBA	Indole-3-butyric acid
LP	Quorin and LePoivre medium
MS	Murashige and Skoog medium
NAA	α-Naphthaleneacetic acid
NS	Nodal segment
PGR	Plant growth regulator
SA	Sodium alginate
SE	Somatic embryo
ST	Shoot tip
WPM	Woody Plant Medium

1 Introduction

Forests which cover barely 7% of the earth's surface harbour over 60% of the world's biodiversity and one of the most valuable eco-systems in the world (Reddy et al. 2018). This biodiversity have not only abundant social and economic tenets but also intrinsic significance. Starting from important ecological functions as soil and watershed protection, forest trees also serve as the source of numerous products which can only be extracted from the forest. Forest trees are the primary raw material base for both of the industrial and domestic wood merchandises, which provide perpetually renewable energy, fibre and timber. Globally, 48% of the forest plantation is established for industrial use and 26% for non-industrial use (fuelwood, soil and water conservation), and the remaining 26% is not specified (Sloan and Sayer 2015). Modern pharmaceutical industry is largely dependent on plant-based drugs; for instance, Indian indigenous tree *Nothapodytes nimmoniana* (*Mappia foetida*) is one of the rare forest species, used for cervical cancer treatment in Japan (Murthy et al. 2018). All of the forests present on earth perform a vital part in climate regulation by acting as the major carbon sink of atmosphere which ultimately prevents the escalation of greenhouse effect. Depending upon their specific utility and human needs, trees are classified under different groups, such as industrial and timber, agroforestry, social forestry, fast-growing nitrogen fixing and multipurpose trees. However, due to rapid deforestation and depletion of genetic resources coupled with escalating human needs, the forest cover is being reduced tremendously from the earth's surface. Some of the direct sources of rapid deforestation are the transformation of forested lands into crop fields, cattle-raising, logging and urbanization, acid rain and fire, mining and oil exploitation, etc. Therefore, it is impossible to meet the increasing demand for forest products along with progressive

deterioration of natural forests both at the same time, and there is an urgent requirement for replacement of natural tree population with cultivated trees (Singh et al. 2017).

Though the conventional approaches such as seed propagation and clonal propagation are being employed for conservation of trees, these are limited to the most valuable and fast-growing species. Several inherent characteristics of forest trees such as slow growth, long life cycle, sexually self-incompatibility and high heterozygosity act as the primary bottlenecks in conventional propagation of trees. High heterozygosity results in higher retention of recessive deleterious alleles within populations, high genetic load and, hence, inbreeding methods such as selfing; backcrossing makes it more challenging to fix anticipated alleles in a certain genetic background (Williams and Savolainen 1996).

Synthetic seed technology has unlocked extraordinary breakthroughs in many areas of basic and applied biological research (Gantait et al. 2015a). Synthetic seed refers to the encapsulation of somatic embryos (SEs) or encapsulated buds, bulbs or any forms of meristem which can develop into plantlets. Encapsulation is usually done in a suitable gel (sodium/calcium alginate) matrix to produce "synthetic seed coat", and the resulting encapsulated propagules can be treated like natural seeds. The facility to incorporate nutrients, biofertilizers, antibiotics or other essential additives to the matrix and the easy handling, storage, transportation and planting are the major advantages to employ synthetic seed technology as a unit of delivery of tissue-cultured plants. Moreover, many trees produce seeds in certain period of the year, whereas synthetic seeds would be available throughout the year. It acts as a simple inexpensive delivery unit of in vitro propagated plants. This review is to highlight the advancements of synthetic seed technology in forest trees.

2 Synthetic Seed

Synthetic seed technology is one of the most important application of plant tissue culture, as it combines the advantages of both clonal propagation and seed propagation (i.e. easy handling, storability, transport, use of sowing equipment and also protection against pests, diseases) (Lambardi et al. 2006). This technology is extensively being used in cryopreservation for long-term storage of plant germplasms (such as the "encapsulation-dehydration" method). This technology emerged from the idea of encapsulating a single SE inside a synthetic seed coat, thus mimicking the natural seeds. Murashige (1977) was the first to produce an official definition of "synthetic seed", "an encapsulated SE, that could be used as a real seed for transport, storage and sowing, and would eventually grow, either in vivo or ex vitro, into complete plantlet". Unlike zygotic embryos, secured by a seed coat and have an access to the nutrients that are accumulated in the cotyledons or in the endosperm, SEs are naked and dependent on the culture medium. Hence, the new concept paved the way for the encapsulation of explants other than SEs and to the formulation of a new definition of synthetic seed (Aitken-Christie et al. 1995) as "synthetically

encapsulated SEs, shoots, or other tissues which can be used for sowing under in vitro or ex vitro conditions".

Encapsulation technology provides many suitable advantages such as easy handling, true to type plants and direct transportation of elite plant materials across the laboratories or greenhouses (Danso and Ford-Lloyd 2003; Naik and Chand 2006). Majority of tree species produces seeds only during particular season of the year, whereas synthetic seeds could be readily available throughout the year (Bapat and Mhatre 2005). It is also an excellent approach for germplasm conservation and storage in sterile condition from short to long duration. However, for long-term storage, slow and steady growth maintenance is most desirable (Englemann et al. 2003). Different methods have been employed in this regard (Gupta and Mandal 2003) such as (1) reduction in temperature and light intensity; (2) application of growth retardants like abscisic acid; (3) employment of minimal growth medium; (4) utilization of osmoticum, for instance, mannitol, sorbitol, etc.; (5) decreased concentration of oxygen; and (6) use of multiple treatment combinations. Long-term conservation of synthetic seeds can also be attained by dipping them in liquid nitrogen (−196 °C), and the procedure is known as cryopreservation. Encapsulation-dehydration and encapsulation-vitrification are two such cryopreservation techniques which are based on basic encapsulation technology. However, somaclonal variation in this regard is a matter of concern (Englemann et al. 2003).

3 Factors Influencing Synthetic Seed Technology

3.1 Type of Explant

A range of explant materials are being efficiently utilized for synthetic seed preparation in forest tree species. Some of the most commonly employed explants are:

3.1.1 Somatic Embryo

SE is considered to be the best suitable explant for synthetic seed production among the different types of propagules. It has a bipolar structure, i.e. it has both shoot and root poles (Standardi and Piccioni 1998). Therefore, it can be formed in bulk which makes them more efficient for encapsulation. SEs can be stored, handled, transported and planted like true seed (Redenbaugh et al. 1993). The employment of SE in development of synthetic seeds in forest tree species has been reviewed and presented in Table 1. SE was effectively utilized for synthetic seed production in several forest tree species, for instance, *Picea abies* (Gupta et al. 1987), *Santalum album* (Bapat and Rao 1988, 1992), *Eucalyptus citriodora* (Muralidharan and Mascarenhos 1995), *Camellia japonica* (Janeiro et al. 1997), *Quercus robur* (Wilhelm et al. 1999), *Feijoa sellowiana* (Guerra et al. 2001), *Goiabeira serrana* (Guerra et al. 2001), *Pinus patula* (Sparg et al. 2002), *Paulownia elongate* (Ipekci

Table 1 Synthetic seed production at their optimal conditions for different forest tree species (in chronological order and alphabetical order)

Species	Explant	Sodium alginate (%)	Calcium chloride (mM)	Duration of storage (weeks)	Survival after storage (%)	References
Picea abies	SE					Gupta et al. (1987)
Santalum album	SE	3	47	6	–	Bapat and Rao (1988)
Betula platyphylla Sukaczev var. *japonica*	Axillary bud	4	100	6	–	Kinoshita and Saito (1990)
Santalum album L.	SE	3	59.25	–	–	Bapat and Rao (1992)
Pistacia vera	Embryoids	3	41	9	14	Onay et al. (1996)
Betula pendula Roth.	ST	2.5–3	–	–	–	Piccioni and Standardi (1995)
Crataegus oxyacantha L.	ST, NS	2.5–3	–	–	–	Piccioni and Standardi (1995)
Camellia japonica L. and *Camellia reticulate* Lindley	ST, NS	–	–	10	10	Ballester et al. (1997)
Camellia japonica L.	SE	3	100	4	40	Janeiro et al. (1997)
Cedrela odorata L.	ST	4	–	48	80	Maruyama et al. (1997)
Guazuma crinita Mart	ST ST, axillary bud	4	–	48	90	Maruyama et al. (1997)
Quercus robur L.	SE	4	–	–	–	Wilhelm et al. (1999)

(continued)

Table 1 (continued)

Species	Explant	Sodium alginate (%)	Calcium chloride (mM)	Duration of storage (weeks)	Survival after storage (%)	References
Hopea parviflora Bedd.	Excised embryo	–	–	30	–	Sunilkumar et al. (2000)
Feijoa sellowiana	SE	1	50	3	81.2	Guerra et al. (2001)
Goiabeira serrana	SE	1	50	10	81.2	Guerra et al. (2001)
Pinus patula	SE	2.2	100 (calcium nitrate)	3	70	Sparg et al. (2002)
Paulownia elongata	SE	3	50	4	43.2	Ipekci and Gozukirmizi (2003)
Cedrela fissilis Vellozo	ST and cotyledonary NS	4	100	42	44	Nunes et al. (2003)
Cedrela fissilis Vellozo	Axillary bud	4	95	24	44	Nunes et al. (2003)
Dalbergia sissoo Roxb.	NS	3	75	–	–	Chand and Singh (2004)
Quercus sp.	Apical bud	4	95	8	90	Tsvetkov and Hausman (2005)
Rauvolfia tetraphylla L.	NS	3	100	4	86.7	Faisal et al. (2006)
Populus tremuloides L. × *P. tremula* Michx. Hybrid aspen	ST	4	100	4	96.7	Tsvetkov et al. (2006)
Acca sellowiana (*O. Berg*) Burret	SE	1	50	6	–	Inocente et al. (2007)
Pinus radiata	SE	3	100	–	73	Aquea et al. (2008)

Elaeis guineensis Jacq.	SE	3	100	4	–	Mariani et al. (2008)
Quercus suber	SE	5	100	8	–	Pintos et al. (2008)
Nothafagus alpina	SE	4–2	100	95	–	Cartes et al. (2009)
Dalbergia sissoo Roxb.	SE	2.5	75	–	43.3	Singh and Chand (2010)
Acacia hybrid	ST and axillary bud	2–5	25–100	6–20	73–100	Asmah et al. (2011)
Corymbia torelliana (F. Muell.) Hill and Johnson. 2x *C. citriodora* (Hook.) Hill and Johnson	ST and NS	3	100	–	–	Hung and Trueman (2012)
Rhododendron dalhousiae	NS	3	60	25	69	Singh and Gurung (2011)
Parkia speciosa Hassk.	Embryo					Ummi et al. (2011)
Eucalyptus hybrid, *C. Torelliana* × *C. citriodora*	ST and NS	3	100	–	76–100	Hung and Trueman (2012)
Drypetes roxburghii (Wall.) Hurursawa	NS	3	100	10–15	90	Murthy and Reddy (2014)
Balanites aegyptiaca Del.	NS	3	100	4	80	Varshney and Anis (2014)
Ficus carica	NS	4	100	1–6	95.6	Sharma et al. (2015)

NS nodal segment, *SE* somatic embryo, *ST* shoot tip

and Gozukirmizi 2003), *Elaeis guineensis* (Mariani et al. 2008), *Pinus radiate* (Aquea et al. 2008), *Quercus suber* (Pintos et al. 2008), *Nothafagus alpine* (Cartes et al. 2009), *Dalbergia sissoo* (Singh and Chand 2010), etc. The major hurdles in the way of producing synthetic seeds using SE as explants are metachronous and delayed development of the embryonic terminal (Castellanos et al. 2004). It was observed that in majority of the plants taken into account had regeneration as low as 50%. Prewein and Wilhelm (2003) even found 26% regeneration in the case of *Quercus robur* synthetic seeds, cultured on P24 medium supplemented with 0.9 μM BA and 0.1 μM IBA. There was only one instance where synthetic seeds developed from SE resulted in 100% regeneration in MS with 2 μM BA plus 0.5 μM IBA (*Hemidesmus indicus*; Cheruvathur et al. 2013). Interestingly, both of these striking differences in regeneration were achieved in different media. Hence, a proficient embryogenic system is indispensable for advancement in plantlet conversion from synthetic seeds.

3.1.2 Shoot Tip

Shoot tips (STs) or shoot buds are the second most amenable explants which are non-embryogenic materials and contain suitable mitotic activity in the meristem (Ballester et al. 1997). The most interesting part of using STs as explants for mass propagation via synthetic seed production is the requirement of space and cost in comparison to conventional ST culture in vitro. The amount of space and culture media needed for multiple shoot formation and its proliferation is 20 times lesser than that of conventional ST culture. It makes the transportation of even bulk of propagules in limited space effortlessly. There has been several reports of ST-utilized synthetic seed production in forest tree species, such as *Cedrela odorata* (Maruyama et al. 1997), *Guazuma crinita* Mart (Maruyama et al. 1997), *Jacaranda mimosaefolia* (Maruyama et al. 1997), *Camellia japonica* and *C. reticulata* (Ballester et al. 1997), *Cedrela fissilis* (Nunes et al. 2003), *Populus tremuloides* × *P. tremula* hybrid aspen (Tsvetkov et al. 2006), Eucalypt hybrid (*C. torelliana* × *C. Citriodora;* Hung and Trueman 2012) and *Eucalyptus urograndis* (*E. grandis* × *E. Urophylla*).

3.1.3 Nodal Segment

The relative simplicity of producing microcuttings or nodal segments (NSs) or axillary buds makes them a fitting explant for synthetic seed production (Piccioni and Standardi 1995). Moreover, better storage potential for genotype exchange, better proliferation after storage and lesser occurrences of physiological variations are the other advantages of utilizing NS for encapsulation (Micheli et al. 2007; Gantait et al. 2015b). NSs were utilized for synthetic seed production (Table 1) in several forest tree species, such as *Betula platyphylla* var. *japonica* (Kinoshita and Saito 1990), *Betula pendula* (Piccioni and Standardi 1995), *Crataegus oxyacantha* (Piccioni and Standardi 1995), *Dalbergia sissoo* (Chand and Singh 2004), *Rauvolfia*

tetraphylla (Faisal et al. 2006), *Acacia hybrid* (Asmah et al. 2011), *Rhododendron dalhousiae* (Singh and Gurung 2011), *Drypetes roxburghii* (Murthy and Reddy 2014), *Balanites aegyptiaca* (Varshney and Anis 2014) and *Ficus carica* (Sharma et al. 2015). In most of the studies, multiple shoot primordia were found to be frequently emerging from synthetic seeds which is another advantage of NS-induced synthetic seeds.

3.1.4 Others

Even though majority of the tree species are more suitable to be preserved from either SEs, NSs or STs, these are not common for all the other tree species. Few of the occasionally used explant materials are apical bud (*Quercus* sp.; Tsvetkov and Hausman 2005), embryo (*Parkia speciosa*; Ummi et al. 2011), embryoids (*Pistacia vera*; Onay et al. 1996), excised embryo (*Hopea parviflora*; Sunilkumar et al. 2000), pregerminated SE (*Acca sellowiana*; Inocente et al. 2007) and cotyledonary NS (*Cedrela fissilis*; Nunes et al. 2003). These studies are the evidences of suitable explant versatility in synthetic seed, prevailing among different types of forest tree species.

3.2 Encapsulating Agents

When looking at the history of synthetic seed technology, a wide number of encapsulating agents have been tested in time for their capacity to produce beads, such as agar, agarose, alginate, carboxymethyl cellulose, carrageenan, ethyl cellulose, gelrite, guar gum, nitrocellulose, Polyox, polyacrylamide and sodium pectate (Datta et al. 2001; Saiprasad 2001). Redenbaugh et al. (1988), after comparing several substances for making synthetic seed coats, proposed the use of a sodium alginate (SA) solution which could be turned into a hardened Calcium (Ca)-alginate gel by an ion-exchange reaction when dropped in the calcium chloride (CC; $CaCl_2.2H_2O$) solution. Bapat and Rao (1988) used alginate matrix for encapsulating SEs of sandalwood. However, the germination rate of synthetic seeds of sandalwood after storage for 45 days at 4 °C was low (10%). Tsvetkov et al. (2006) achieved 96% germination recovery in alginate-encapsulated ST of hybrid aspen after 4 weeks of storage at 4 °C. In most of the forest tree species, the same encapsulating agents (SA and CC = Ca-alginate matrix) at different combinations of concentration were used for synthetic seed preparation (Table 1). The main advantages of this compound are the excellent water solubility and moderate viscosity of SA at room temperature, its easy availability at low cost, the long-term storability of the SA solution, the easy use of calcium salts for rapid gelation and bead hardening at room temperature, the possibility to prepare synseeds of different hardness by changing the concentration of SA and/or the duration of the ion-exchange reaction, the absence of any kind of toxicity of the Ca-alginate matrix for explants and easy incorporation of alginate in nutritive medium to obtain an synthetic endosperm. However, high adhesion and rapid desiccation of Ca-alginate beads in presence of air

make them difficult to handle and hinder the shoot-root emergence from encapsulated explants. To resolve these issues, Redenbaugh et al. (1987) pointed out the application of a hydrophobic layer at the surface of the synthetic seeds. Several hydrophobic coating agents have been tested over time, such as aluminium monostearate, Elvax 4260 (Dupont, SRI Int.), Gantrez ES, glutaraldehyde, methyl vinyl ether/maleic anhydride, polylysine and polyproline in this regard (Redenbaugh et al. 1988). Among them, Elvax 4260 exhibited a substantial impediment to capsule drying. Khor et al. (1998) introduced the concept of a non-tacky, water barrier coating of the alginate capsules, obtained by means of alginate chitosan or alginate-gelatin encapsulation which simulates the endosperm of seeds, better protection and easy handling.

3.3 Polymerization and Encapsulation Matrix

Numerous factors are involved in maintaining encased explant like temperature, humidity and nutrient reservoir in order to provide protection and optimum germination. Evidently, matrix resources play the chief function in sustaining the germination viability of the biological material (Gantait et al. 2017). The SA-coated explants are dropped singly into the polymerizing/complexing agent, primarily CC and kept for 20–25 min for the formation of ideal beads. The encapsulated seed rigidity depends on the amount of Na^{+}ions present in SA solution exchanged with the Ca^{2+} ions of CC solution; as a result the polysaccharide molecules form a polymeric structure producing a bead of Ca-alginate (Daud et al. 2008). Accordingly, the matrix attains the required consistence to supply security against desiccation and mechanical injuries. Majority of the reports employed 3% SA and 100 mM CC to facilitate proper ion exchange thus produces the most advantageous bead formation (Table 1).

3.4 Duration of Polymerization

Synthetic seed formation is largely regulated by the time of SA and CC polymerization. The optimum time period for polymerization is 20–30 min within which the Ca-alginate beads are symmetrical in shape (Asmah et al. 2011). Longer polymerization time (30 min) increases the adsorption of Ca^{2+} by SA since it is occurred through surface. Though it gives hardier texture of beads and better protection during handling and transportation, at the same time it suppresses regeneration due to Ca ion toxicity and also for shoot and root emergence (Nagesh et al. 2009). Hence, it is necessary to wash the hardened beads several times with sterile water to remove the toxic remnants of chloride and sodium ions (Gantait et al. 2015a). After washing, the beads or encased explants should be blotted on blotting paper, and either can be stored or relocated to regrowth medium for subsequent regeneration.

4 Regrowth Assessment

After the completion of last encapsulation steps, regeneration of the encapsulated material is essential. To achieve this, basal media in different concentrations such as ¼-, ½- and full-strength Murashige and Skoog (1962) (MS), Quoirin and LePoivre (1977) medium (LP), Woody Plant Medium (WPM) (Lloyd and McCown 1980) or any nutrient medium can be used for both of their semi-solid and liquid states. However, the germination rates vary significantly with different media compositions (Inocente et al. 2007). Most of the researchers have found full MS to be most suitable for shooting and regeneration whereas half MS for rooting and acclimatization. The most possible causes for the variable effects of nutrient strengths of the regrowth media on different tree species can be due to higher nutrient toxicity or nutrient deficiency. Varshney and Anis (2014) achieved 80% shooting from encapsulated NSs of *Balanites aegyptiaca* Del. in MS medium supplemented with 12.5 μM BA and 1.0 μM NAA. However, 70% rooting was observed in half MS supplemented with 1.0 μM IBA. Singh and Gurung (2011) observed better conversion of encapsulated STs in plantlets in MS medium (69.5%) containing activated charcoal (1.0%, w/v) and 1.0 μM IBA in comparison with half MS containing the same (51.75%). According to Singh and Chand (2010), inaccessibility of encapsulated plant tissue to the nutrients is one of the limiting factors for the germination. So, endogenous or exogenous supply of nutrients is mandatory for the SA-encapsulated meristematic plant tissue to improve the rate of germination. Inversely, in many reports, the supplementation of plant growth regulators (PGRs) in the regrowth medium boosts the germination of synthetic seeds. Supplementation of PGRs to the regrowth medium has been found to abolish the obligation of a supplementary in vitro root induction step before acclimatization.

5 Application of Synthetic Seeds

Synthetic seed technology has unravelled pioneering outlooks on the ground of plant biotechnology. Various approaches can be taken using synthetic seeds that could assist managing the plant germplasm and further discussed in the sections below.

5.1 Direct Sowing

Direct sowing of encapsulated seeds in ex vitro condition is commercially more viable process and in turn low-cost practice for the regrowth of plantlets (Kundu et al. 2018). Sowing of the synthetic seeds directly in the soil skips the acclimatization procedure that is unexceptionally required for micropropagated plants. Synthetic seeds can be sown directly in ex vitro planting substrates as perlite, sand,

soilrite, vermicompost, etc. for the commercialization of the process (Mandal et al. 2000). Ex vitro regeneration has been not been effectively tried except for only few researchers. Ipekci and Gozukirmizi (2003) used soil containing peat and perlite (3:1) for direct sowing of *Paulownia elongata* encapsulated SEs and recorded 43.2% of germination frequency. Chand and Singh (2004) found 45% regeneration of *Dalbergia sissoo* synthetic seeds on peat moss supplemented with ½-strengthMS medium. The major hurdles faced during direct sowing of synthetic seeds were the contamination by microbes. The stored nutrients and exudates emitted by the encased explants are particularly accountable for the infection. Incorporation of fungicides as carbendazim or bavistin and benomyl at 50–100 mg l^{-1} into the encapsulation matrix can resolve this issue to certain extent (Bapat and Rao 1993). Antonietta et al. (2007) obtained 96.7 and 100% regeneration of encapsulated *Citrus reticulata* on filter paper and perlite, correspondingly only after incorporation of 100 mg l^{-1} thiophanate-methyl into the encapsulation matrix. Sometimes, fungicides may also affect the viability of synthetic seeds and directly influence regeneration.

5.2 Short-Term Storage

Commercial utilization of synthetic seed technology predominantly requires flexibility in storage and efficient regeneration even after storage. Thus, suitable conditions and durations for storage are necessary to utilize this technique for commercial purpose (Gantait and Kundu 2017). Most commonly found suitable temperature for storage of synthetic seed in tree species is 4 °C (Singh and Gurung 2011; Sharma et al. 2015). Sandalwood was one of the first tree species where embryogenic cell suspensions were used for encapsulation and storage at 4 °C for 45 days (Bapat and Rao 1988). Palanyandy et al. (2015) observed that synthetic seeds of oil palm polyembryoids stored at 5 °C for 60 days had higher survival rate than those stored at room temperature (25 °C) that lost viability after 30 days of storage. Ipekci and Gozukirmizi (2003) observed that the germination frequency in encapsulated embryos of *Paulownia elongata* after 60-day storage (32.4%) was lower than that after 30-day storage (43.2%) at 4 °C. There are some instances where higher-temperature storage provides better regeneration rate. Maruyama et al. (1997) attempted above freezing temperatures for storage of encapsulated STs in three different tropical forest tree species. The post-storage viability was 80% after 12 months at 12 °C for *C. odorata*, 90% after 12 months at 25 °C for *G. crinita* and 70% after 6 months at 20 °C for *J. mimosaefolia*. Synthetic seeds of plantation eucalypt (*Corymbia torelliana* × *C. Citriodora*), containing explants pretreated with IBA, were stored for 8 weeks, and the regrowth frequencies were 50–84% at 25 °C storage whereas 0–4% at 4 °C storage (Hung and Trueman 2012). Nunes et al. (2003) obtained 96–100% regeneration in encapsulated STs and cotyledonary NSs stored at 25 °C in *Cedrela fissilis* Vellozo.

5.3 *Long-Term Storage*

Generally, cryopreservation techniques are followed for long-term preservation of germplasms. At this ultra-low temperature, biochemical and physiological mechanisms of the cell become ceased without undergoing any damaging effect. This technique is successful if the intracellular ice crystallization is by passed since it damages the cell membrane, effecting semipermeability. Thus far, a range of approaches have been made for cryopreservation of plant tissues, they are the two-step freezing, simple desiccation, vitrification, encapsulation-dehydration and encapsulation-vitrification. There is no report available on long-term conservation; hence, here lies the prospect of research.

6 Limitations

Although results of intensive researches in the field of synthetic seed technology seem promising for propagating a number of forest tree species (Table 1), practical implementations of this technology are constrained due to the following main reasons:

1. Limited production of viable micropropagules useful in synthetic seed production
2. Anomalous and asynchronous development of SEs
3. Improper maturation of the SEs that makes them inefficient for germination and conversion into normal plants
4. Lack of dormancy and stress tolerance in SEs that limit the storage of synthetic seeds
5. Poor conversion of even apparently normally matured SEs and other micropropagules into plantlets that limit the value of the synthetic seeds and ultimately the technology itself

Most of the tree species studied for encapsulation was subjected to drastic or moderate reduction in regeneration after encapsulation. It is mainly due to asynchronic maturation of embryonic pole, which makes the downstream processes challenging (Tapia et al. 1999; Castellanos et al. 2004, Cartes et al. 2009). Several efforts have been made to improve germination and conversion of encapsulated seeds to plants in woody species such as use of different gelling agents (agar, gelrite) for encapsulation, alteration in SA concentration, tweaking polymerization time, etc. (Patel et al. 2000; Maruyama et al. 2003; Utomo et al. 2008). Aquea et al. (2008) found very less reduction in germination, i.e. from 73% (nonencapsulated) to 66% (encapsulated) SEs of *Pinus radiate*. Cartes et al. (2009) achieved higher levels of germination in Nothofagus alpine by using zygotic embryos (100%) in comparison with the SEs (45%), owing mainly to the lack of maturity and synchrony of the embryoids.

A number of researchers have also approached to develop the quality (Guerra et al. 2001; Ipekci and Gozukirmizi 2003; Inocente et al. 2007; Aquea et al. 2008;

Singh and Chand 2010) of SEs by reforming the culture medium composition (type and strength), growth regulators (types and concentrations), physical state of the medium (solid, liquid), incubation conditions (temperature, illumination), etc. Attree and Fowke (1993) and Fowke and Attree (1996) have described that inclusion of high levels of sucrose (i.e. permeating osmotica) and ABA (which is associated with water stress) in the standard medium prevents maturation, while inclusion of PEG (non-permeating osmotica) with ABA dramatically improves the frequency and synchrony of the SE maturation in spruce. Presence of sucrose enhances the viability of synthetic seed and their subsequent development, maturation and germination in many plant species (Jain et al. 1995; Attree et al. 1995; Fowke and Attree 1996). ABA regulates somatic (Lee and Soh 1994) and zygotic (Crouch and Sussex 1981; Ammirato 1983) embryo maturation (Norstog 1965; Choi et al. 1997; Liu et al. 1993). Nevertheless, desiccation and subsequent rehydration have been found useful in inducing a high-frequency conversion of SEs into plantlets in some species (Gray 1987; Compton et al. 1992; Redenbaugh 1990; Redenbaugh et al. 1993).

7 Conclusion

Synthetic seed technology is undoubtedly a breathtaking biotechnological tool with profound potential in modern times. Our current review summarizes the most commendable findings in synthetic seed production of various forest tree species. However, if we compare the progress of this technology in tree species with other plant species, it becomes clear that only a handful number of tree species are being studied for decades. There has not been any significant modification in the technology from the early days of its development. Though, the practical utility of this technology mostly depends on the economic values of the concerned plant, it is imperative to conserve all of the endangered and also commonly found forest tree species. Hopefully, the policy makers and research around the world will be able to utilize synthetic seed technology in future.

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Medium- and Long-Term Conservation of Ornamental Plants Using Synthetic Seed Technology

Hamit Ekinci, Jayanthi Nadarajan, and Yelda Özden Çiftçi

Abstract Synthetic seed (synseed) describes artificially encapsulated plant tissues, usually somatic embryos but also other vegetative parts that can be propagated into complete plants under in vivo or in vitro conditions. Synseed technology can be utilised for medium-term storage and long-term conservation of valuable ornamental plant germplasm. Synseeds can be conserved in vitro for several years through maintenance of encapsulated propagules at low temperatures (slow growth storage technique) or they can be preserved theoretically ad infinitum at the ultra-low temperatures of liquid nitrogen. In this chapter, we review recent studies in the conservation of various ornamental plant species using synseeds developed from different plant explants (i.e. somatic embryos, protocorm-like bodies, shoot tips, bulblets and axillary buds).

Keywords Artificial seed · Encapsulation-dehydration · Encapsulation-vitrification · Slow growth storage · Synseed · Cryopreservation

1 Introduction

Ornamental plants comprise a huge diversity of genera, species, and cultivars. Plants are propagated sexually and asexually. There is significant international trade in ornamental plants, estimated at US$55 billion in 2016 (Sources: UN-Comtrade, Royal Flora Holland, Rabobank, 2016). The consumption of flowers and pot plants as ornamentals is forecast to reach 100 billion dollars by 2027 (Van Horen 2017).

H. Ekinci · Y. Ö. Çiftçi (✉)
Department of Molecular Biology and Genetics, Gebze Technical University, Kocaeli, Turkey
e-mail: ozden@gtu.edu.tr

J. Nadarajan (✉)
The New Zealand Institute for Plant and Food Research Limited, Palmerston North, New Zealand
e-mail: Jayanthi.nadarajan@plantandfood.co.nz

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Many ornamental varieties are clonally propagated with in vitro tissue culture is considered the most efficient ornamental plant propagation method (Kulus 2016).

Clonal propagation is only feasible if plants can be sold at a price that justifies the higher cost of propagation. Higher priced plants come with expectations of quality, uniformity and health status. In vitro growing and propagation of plants provide opportunities to deliver plants that meet these requirements. The in vitro environment additionally provides opportunities to maintain the 'mother' plants from which subsequent generations of invite cultures are propagated. Additionally as for other plants, the species from which ornamental cultivars are developed face extinction threats due to over-collection, urbanization, climate change and habitat destruction. For this purpose, traditional in situ and ex situ conservation strategies must be developed to complement with biotechnological preservation techniques to safeguard and yield high-quality horticultural productions (Benelli et al. 2017).

In this chapter, we review the use of synthetic seed technology for the conservation of ornamental plants for medium- to long-term.

2 Concepts of Conservation

The general strategies to conserve plant biodiversity including ornamental plants are in situ conservation, ex situ conservation, sustainable use and legislation. Nature reserves (or protected areas or parks) are an important tool for in situ conservation of ornamental species biodiversity. However, this can be expensive requiring professionally skilled staff and implementation of laws (Long et al. 2018). Another form of in situ conservation for ornamental plant germplasm is to maintain them in traditional agroecosystems (or on-farm conservation).

Botanical gardens are the most common method of ex situ ornamental germplasm conservation. However, ex situ conservation of ornamental species as field and greenhouse collections requires high maintenance, including skilled labour and is expensive (Reed 2006). In some cases, it is not germplasm in the traditional sense that requires conservation but high-value breeding lines such as doubled haploid or transgenic genotypes (Rajasekharan et al. 1994; Joung et al. 2006). In both in situ and ex situ outdoors growing situations, plants are exposed to environmental factors (e.g. biotic and abiotic stresses). Adopting in vitro strategies can address these problems: typically, through plant tissue culture techniques, where plant organs, cells or tissues are used to produce new plant cells, tissues or metabolites in aseptic culture conditions. With this approach, it is possible to conserve and produce virus-free (e.g. meristem culture), genetically stable and clonally produced ornamental plants in high quantities (Engelmann 2011). A risk with this approach is the somaclonal variation frequently reported in in vitro propagation systems.

Somaclonal variation has been reported at different levels (morphological, cytological, cytochemical, biochemical and molecular) in various micropropagated crops (Do et al. 1999; Mujib 2005; Orbovic et al. 2008; Sanchez-Teyer et al. 2003; Cooper et al. 2006; Biswas et al. 2009; Jin et al. 2008; Bednarek et al. 2007;

Van den Bulk et al. 1990; Gao et al. 2009; Sharma et al. 2007; Ahmed and Abdelkareem 2005). Though extreme levels of somaclonal variation between 100% (Orton 1984) and 90% in banana (Smith and Drew 1990) have been reported, typically an average of 15–20% can be expected (Skirvin et al. 1994) depending on the number of culture cycles and culture conditions. The levels of somaclonal variation can vary with genotype, type of tissue, explant source, media components and the duration (and number of) of the culture cycle (Pierik 1988). Though somaclonal variation is inevitable in tissue culture, manipulating, controlling and minimizing the putative inductive factors mentioned above will reduce the level of occurrence.

Synthetic seed technology offers complementary approaches for preserving ornamental plant germplasm in two ways: medium-term (in vitro storage) by incubating cultures in sub-optimal growth conditions (e.g. darkness, low temperature or high sugar content in growing medium) and long term (cryopreservation) by suspending all metabolic activities through storage in ultra-low temperature of liquid nitrogen (LN, −196 °C) (Ozudogru et al. 2010; Akdemir et al. 2010). These approaches to conservation are discussed in detail below.

3 Usage of Synthetic Seeds for Conservation

Synthetic seeds (synseeds), described as 'artificial seeds' by Murashige (1977) are artificially encapsulated somatic embryos (usually) or other vegetative parts of plants such as shoot tips, cell aggregates, axillary buds, nodal segments, protocorm-like bodies (PLBs) or any other micropropagules, which can be used as seed and grown into a plant under in vitro or in vivo conditions (Rihan et al. 2017). A technique for hydrated encapsulation, using calcium alginate (termed 'hydrogel'), was developed by Redenbaugh et al. (1986). The encapsulating hydrogel can be engineered to perform a similar function to that of the seed coat and endosperm of normal seed providing physical protection and carrying compounds such as nutrients, plant growth regulators, antibiotics and fungicides to assist germination and plant growth (Gray et al. 1991). Synseeds have applications across hybrids, non-seed species and elite genotypes offering many benefits such as pathogen-free plant production, genetic uniformity, low-cost and rapid plant production, plus germplasm protection (Rihan et al. 2017).

Synseed technology is already used across a range of plant species, including vegetables, fruits, medical plants, ornamentals, forest trees, orchids and cereals for production and conservation (i.e. Ara et al. 2000; Sharma and Shahzad 2012; Rihan et al. 2011; Ahmad and Anis 2010; Rai and Jaiswa 2008; Rai et al. 2009; Danso and Ford-Lloyd 2003; Ozden Tokatli et al. 2008; Akdemir et al. 2010; Ganapathi et al. 1992; Mandal et al. 2000; Nyende et al. 2003; Chand and Singh 2004; Singh et al. 2009; Micheli et al. 2007; Faisal and Anis 2007). Synthetic seed technology is especially useful for the propagation of rare hybrids and elite genotypes (Mandal et al. 2000; Bukhari et al. 2014). Although the majority of artificial seeds

are produced using in vitro-derived propagules, synseeds have also been produced from in vivo-derived propagules (Sharma et al. 2013).

Establishment of a somatic embryogenesis pathway is a prerequisite for artificial seed production. However, there are many challenges particularly for species that are recalcitrant to somatic embryogenesis. In this situation, non-embryogenic propagules can be used for the production of artificial seeds but require additional technique development, for example optimised rooting systems. Limitations in storage of synseeds are reported due to lack of dormancy, non-synchronised maturation, low-level of conversion into plantlets and reduced viability following storage at low temperatures (Makowczynska and Andrzejewska-Golec 2006).

4 Medium-Term Conservation

The main purpose of protecting plant germplasm using in vitro approaches is to preserve the genetic integrity of a genotype(s) in an environment away from the field risks of biotic and abiotic factors, while limiting the number of subcultures without endangering the plant material (Shibli et al. 2000; Moges et al. 2003). Encapsulated plant propagules in artificial seeds can be stored in vitro for medium-term durations (1–2 years) under growth-limiting or sub-optimal conditions (West et al. 2006). Similar techniques to those used to reduce growth in vitro such as incubation at low temperature, and/or low light intensity, manipulation of nutrient components in the medium, use of osmotic agents and growth retardants can be applied for artificial seed storage as well (Tahtamouni et al. 2001; Moges et al. 2003).

The most common method used to reduce the growth of tissues in cultures is to lower the temperature of cultures, avoiding temperatures below freezing or where freezing injuries may occur (Gull et al. 2019; Lyons et al. 1979). For artificial seed storage reductions in temperature and light intensity can be used simultaneously. The basic principle here is that incubation at a lower temperature will reduce metabolic activity and thus limit the growth of encapsulated plant materials. In most cases, the optimum storage temperature and light intensity are species specific. For instance, artificial seeds derived from nodal buds of *Hibiscus moscheutos* have been successfully preserved at 5 °C for 36 months (West et al. 2006) while artificial seeds of somatic embryos of *Pistacia vera* L. (pistachio) were preserved at 4 °C for up to 2 months (Onay et al. 1996). In a study conducted by Ozden Tokatli et al. (2008), synseeds of photinia shoot tips were conserved at 4 °C for 3 months with 90% showing 'germination' after storage.

Incubation with osmotic agents at room temperature or at low temperature can also be used to prolong the storage period of artificial seeds. The change of carbon source is known to have a remarkable effect on growth rate (Rajasekharan and Sahijram 2015) by reducing the water potential of plant cells. The addition of osmotic substances to the culture medium is effective in retarding growth and increasing the viable storage time of many artificial seeds of different plant species in vitro (Shibli 1991). Osmotic agents such as mannitol, sucrose, sorbitol

(Shibli et al. 1992), tributyl (2,4-dichlorobenzyl) phosphonium chloride (Posfon D), maleic hydrazide, succinic acid-2, 2 dimethyl hydrazide (B-995), CCC (2-chloroethyl trimethylammonium chloride) and ancymidol (Dodds and Roberts, 1985) are among commonly used chemicals for medium-term storage of artificial seeds.

Growth-retarding compounds that are used to reduce the overall growth rate of in vitro plantlets and thereby increase the subculture intervals can also be applied to artificial seed storage. The choice of growth inhibitor generally depends on the growing conditions and can be species specific but offers a simple, efficient and potentially cheap method of storage since the artificial seeds can be stored using these chemicals in a standard rather than specialised growth rooms. However, growth retardant use has some attendant problems: (1) the plant may be stunted and show abnormal growth, so it may be difficult to regenerate normal plants; (2) the presence of these retardants can lead to resistance to growth retardants or the development of tolerant lines and (3) since some of the growth retardants have mutagenic properties there may be genetic changes (Rajasekharan and Sahijram 2015). Hence, careful consideration is needed when selecting growth-retarding compounds for a particular genotype to mitigate the above stated problems.

Some examples of artificial seed medium-term storage are described here. In a study by Ozden Tokatli et al. (2008), axillary buds taken from in vitro propagated shoots of photinia (*Photinia fraseri*) synseeds prepared using sodium alginate (Na-alginate) (3%) were stored for 3 months at 4 °C and showed high viability (100%) and regeneration (91%) after storage. A study by Akdemir et al. (2010) showed that encapsulated shoot tips of photinia could be maintained up to 6 months at 4 °C in dark with 91.6% sprouting on MS medium. Different explants of various ornamental plant species have been successfully stored for a range of durations in the form of synseeds (Table 1).

5 Long-Term Conservation (Cryopreservation)

Cryopreservation, which is preservation of viable cells, tissues, organs and organisms at ultra-low temperatures (c. −196 °C) in the liquid or vapour phase of liquid nitrogen, is used for long-term conservation of valued plants. This method of preservation is growing in popularity for plant germplasm conservation due to its comparatively low maintenance cost, small space requirement and its reliability (Sakai 2004). The advantages of cryopreservation over slow-growth storage or in vitro storage are the reduced threats of contamination and somaclonal variation (Engelmann 2011). Cryopreservation also provides the opportunity to conserve what is otherwise considered as difficult to store species such as those with recalcitrant seeds, and vegetatively propagated plant germplasm (Touchell 2000). This method of preservation is now well established for ornamental species and has been previously reported for species such as *Dianthus hybrida* (Fukai 1989), *Chrysanthemum* × *grandiflorum* (Fukai 1989) and various other species (Kulus and Zalewska 2014).

Table 1 The recent applications of synseeds on different types of ornamental plants for medium-term conservation

Species	Plant explant	Encapsulation procedures	Storage condition and duration	Recovery (%)	References
Camellia japonica	Somatic embryos	3% sodium alginate, 100 mM $CaCl_2$ solution, 30 min for complete polymerization of alginate Transferring to MS with 3% sucrose and 4.44 µM BA and 0.41 µM IBA	4 °C in darkness for 2 months	30	Janeiro et al. (1997)
Cedrela fissilis	Shoot tips, cotyledonary and epicotyl nodal segments	4% sodium alginate, 1.4% $CaCl_2$ solution, 30 min for complete polymerization of alginate Transferring to MS with 2% sucrose and 2.5 µM BA	25 °C in16 h (20–25 µmol m^{-2} s^{-1}) for 9 months	6–8	Nunes et al. (2003)
Drimiopsis kirkii	Embryogenic callus	4% sodium alginate, 3% $Ca(NO_3)_2$ solution, 15 min for complete polymerization of alginate Transferring to MS with 3% sucrose without growth hormones	15 °C in 16 h (3000 lux) for 4 months	64.4	Haque and Ghosh (2014)
Hibiscus moscheutos (cv Lord Baltimore)	Nodal segment	2.75% sodium alginate, 50 mM $CaCl_2$ solution, 20 min for complete polymerization of alginate Transferring to DKW with 3% sucrose and 10^{-7} M TDZ	5 °C in darkness for 36 months	80	West et al. (2006)
Metrosideros excelsa Soland. ex Gaertn.	Apical buds	3% sodium alginate, 100 mM $CaCl_2$ solution, 30 min for complete polymerization of alginate Transferring to MS with 3% sucrose and 0.88 µM BA	4–10 °C in darkness for 4 months	58	Benelli et al. (2017)
Morus spp.	Axillary buds	2–8% sodium alginate, 25–100 mM $CaCl_2$ solution, 30 min for complete polymerization of alginate Transferring to MS with 3% sucrose and 4.4 µM BA	5 °C in darkness for 3 months	18	Pattnaik and Chand (2000)

Nerium oleander	Shoot tips	3% sodium alginate, 100 mM $CaCl_2$ solution, 30 min for complete polymerization of alginate Transferring to MS with 3% sucrose without growth hormones	4 °C in darkness for 3 months	75	Ozden-Tokatlı et al. (2008)
Paulownia elongata	Somatic embryos	3% sodium alginate, 50 mM $CaCl_2$ solution, 30 min for complete polymerization of alginate Transferring to MS with 3% sucrose without growth hormones	4 °C in darkness for 2 months	32	Ipekci and Gozukirmizi (2003)
Photinia × *fraseri* Dress	Apical buds	3% sodium alginate, 100 mM $CaCl_2$ solution, 30 min for complete polymerization of alginate Transferring to QL with 3% sucrose and 4.4 μM BA	4–10 °C in darkness for 4 months	65	Benelli et al. (2017)
Photinia fraseri	Shoot tips	3% sodium alginate, 100 mM $CaCl_2$ solution, 30 min for complete polymerization of alginate Transferring to MS with 3% sucrose without growth hormones	4 °C in darkness for 3 months	91	Ozden-Tokatlı et al. (2008)
Polygala myrtifolia	Apical buds	3% sodium alginate, 100 mM $CaCl_2$ solution, 30 min for complete polymerization of alginate Transferring to DKW with 3% sucrose and 8.8 μM BA	4–10 °C in darkness for 8 months	68.8	Benelli et al. (2017)
Rosa hybrida 'King's Ransom'	Somatic embryos	3% sodium alginate, 100 mM $CaCl_2$ solution, 30 min for complete polymerization of alginate Transferring to MS with 3% sucrose and 1.0 mg/L BAP and 0.1 mg/L NAA	4 °C in darkness for 40 days	30	Jayasree and Devi (1997)
Splachnum ampullaceum	Moss buds	1% sodium alginate, 100 mM $CaCl_2$ solution, 20 min for complete polymerization of alginate Transferring to Heller's with 3% sucrose without growth hormones	5 °C in darkness for 30 months	50	Mallón et al. (2007)

(continued)

Table 1 (continued)

Species	Plant explant	Encapsulation procedures	Storage condition and duration	Recovery (%)	References
Syringa vulgaris	Axillary buds	2% sodium alginate, 50 mM $CaCl_2$ solution, 30 min for complete polymerization of alginate Transferring to MS with 3% sucrose and 5 mg/L BA and 0.01 mg/L NAA	5 °C in darkness for 45 days	83	Refouvelet et al. (1998)
Rauvolfia tetraphylla L.	Nodal segments	3% sodium alginate, 100 mM $CaCl_2$ solution, 30 min for complete polymerization of alginate Transferring to MS with 3% sucrose and 10 µM BA, 0.5 µM NAA	4 °C in darkness for 4 weeks	86.7	Faisal et al. (2006)
Rauvolfia serpentina	Shoot tips	3% sodium alginate, 100 mM $CaCl_2$ solution, 45 min for complete polymerization of alginate Transferring to MS with 3% sucrose without growth hormones	4 °C, 1.5 µmol m^{-2} s^{-1} for 14 weeks	68.5–100	Ray and Bhattacharya (2008)

Cryopreservation protocols are usually developed empirically for specific materials or explants taking into consideration the physiological and biophysical factors of the explants to minimise stress and maximise survival (Nadarajan and Pritchard 2014). In addition to this, cryoprotection applied in the form of osmo-protection or chemical cryoprotection, is the most important factor in determining successful post-cryopreservation survival (Benson 2008). The development of a simple and reliable cryopreservation protocol would allow wider application of this preservation technique in the conservation of plant materials. Desiccation-tolerant explants such as orthodox seeds and dormant buds can be cryopreserved without complicated pre-conditioning and pre-culture treatments as cryopreservation mimic the dehydration process they go through in their natural lifecycle (Pritchard and Nadarajan 2007; Engelmann 2011). In contrast, explant materials such as cell suspensions, calluses, shoot tips and embryos that typically contain high amounts of cellular free water are extremely susceptible to freezing injury. Therefore, they may need to be dehydrated artificially to prevent intracellular ice crystallization and cell death (Engelmann 2011).

Classical cryopreservation techniques are based on freeze-induced dehydration achieved through controlled cooling rates—these must neither be too slow, which risks losing water from the cell, nor too rapid, which risks the formation of intracellular ice (Engelmann 2004). The cooling rate follows the well-known inverted 'U' shape where the cell membrane acts as a physical barrier and prevents the ice seeding formation within cell membranes as they are super-cooled (Mazur 2004). Controlled slow cooling techniques usually comprise a sequence of cryoprotection steps namely, pre-culture, stepwise cooling and then osmo-protection with a combination of cryoprotectants (Benson 2008). This technique has been applied successfully to cryopreserve cell suspensions and callus (Kartha and Engelmann 1994; Withers and Engelmann 1998).

Modern cryopreservation techniques involve vitrification, a physical process by which a concentrated aqueous solution solidifies into a stable amorphous glass without the formation of ice crystals when the temperature is decreased (Sakai 2004). Vitrification of plant specimens can be achieved in a number of ways, including desiccation and more recently through the use of highly concentrated plant vitrification solutions (PVS) that readily form glasses on cooling and inhibit crystallization (Sakai 2004; Taylor et al. 2004). Vitrification-based methods typically involve pre-treatment of samples with concentrated cryoprotectant solution: on rapid freezing a highly viscous solid 'glass' forms, thus avoiding lethal ice injury. A vitrification protocol has the following successive steps: pre-growth of mother plants; pre-culture of explants; treatment (loading) of samples with cryoprotective substances; dehydration with highly concentrated vitrification solutions; rapid cooling and rewarming; removal of cryoprotectants (unloading) and recovery (Reed 2008). The vitrification solutions most commonly employed for freezing plant tissues and organs have been termed plant vitrification solutions (PVS). PVS combine cryoprotectants that vary in permeability [e.g. dimethyl sulphoxide (DMSO) and glycerol], such that cellular water is replaced, cell viscosity is increased

and the freezing behaviour of the remaining water is altered (Muldrew et al. 2004; Volk and Walters 2006).

5.1 *Encapsulation Cryopreservation*

Advancement in cryobiological studies and increased demand for tailored cryopreservation protocols led to the development of another innovative cryopreservation technique, encapsulation-dehydration, in the early 1990s. This procedure is based on the technology developed for the production of artificial seeds (Redenbaugh et al. 1986) and the premise that alginate encapsulation should allow tissues to better withstand physical manipulation (Yap et al. 1998). This technique was established for cryopreservation of *Solanum* shoot tips (Fabre and Dereuddre 1990; Dereuddre et al. 1991). The two main techniques adapted in encapsulation cryopreservation protocol (encapsulation-dehydration and encapsulation-vitrification) are discussed below.

5.1.1 Encapsulation-Dehydration Cryopreservation

Encapsulation-dehydration cryopreservation is a stepwise process where explants are preconditioned, encapsulated in alginate beads, osmo-protected by pre-culture in sucrose-enriched liquid or on solid medium, and partially desiccated in the air current of a laminar airflow cabinet or with silica gel and then rapidly frozen in liquid nitrogen followed by rapid warming in a water bath at 40 °C (Fig. 1). Post-cryopreservation survival rate following this technique is reported to be high and fast regeneration compared to other cryopreservation protocols used (Sakai et al. 2000).

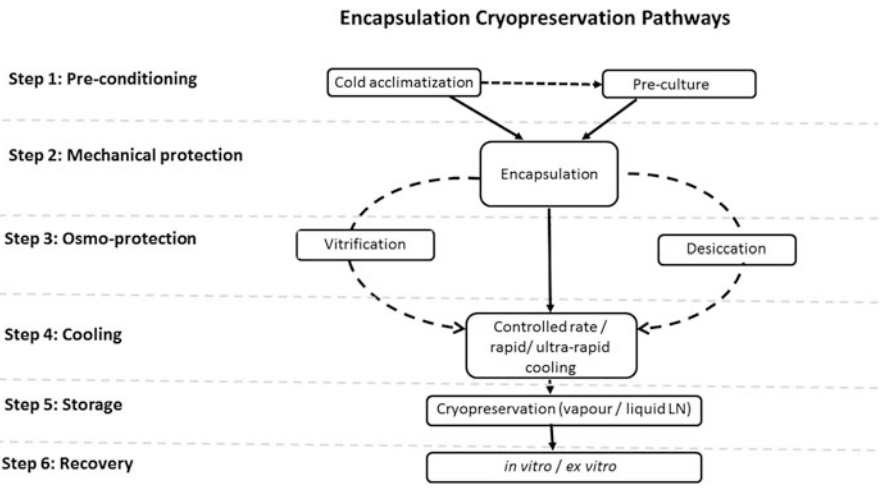

Fig. 1 Steps involved in encapsulation cryopreservation pathways

In 2000, a modified protocol was proposed involving simultaneous encapsulation and pre-growth in medium with sucrose and glycerol (Sakai et al. 2000). A further modified encapsulation-dehydration cryopreservation protocol replacing cold acclimation with high sucrose concentration pre-culture was developed by Reed et al. (2005). Numerous ornamental species, including temperate and tropical species, have been cryopreserved using this technique as apices, embryonic axes, cell suspension cultures, and root cultures (Table 2).

5.1.2 Encapsulation-Vitrification Cryopreservation

Although the encapsulation-dehydration cryopreservation has been successfully applied to various tissues of many species, the requirement to develop a simple and reliable cryopreservation method that was widely applicable remained. This led to the development of encapsulation-vitrification cryopreservation combining both encapsulation-dehydration and vitrification by use of addition vitrification solutions (Matsumoto et al. 1995; Sakai et al. 2008; Fig. 1). This technique has proved particularly useful for highly desiccation-sensitive materials such as shoot tips and meristematic tissues as vitrification enables maintenance of viability of hydrated cells and tissues while avoiding lethal intracellular ice crystallization during the cryopreservation procedures. Exposure time to PVS2 is a critical step in successful cryopreservation by vitrification: there is a compromise between sufficient cellular dehydration and limiting injury from chemical toxicity or excessive osmotic stress during treatment with PVS2 (Sakai et al. 2008; Nadarajan and Pritchard 2014). The encapsulation-vitrification cryopreservation technique has been successfully applied to many ornamental species (Table 2).

5.2 Potential Application of Encapsulation Cryopreservation for Various Tissue Types

Encapsulation cryopreservation is a versatile technique that can be used to cryo store many different plant tissues including shoot tips, somatic embryos, microspore embryos, ovules, PLBs, calluses, cell suspension cultures, hairy root cultures and microalgae (Engelmann et al. 2008; Pence 2014). Vegetative tissue cryopreservation is particularly useful when dealing with species that have extremely desiccation-sensitive seeds, produce no seed or have seeds, embryos or embryonic axes that cannot be adapted to cryopreservation (Pence 2014). Logistically this is often an easier option than cryopreserving large and highly metabolically active recalcitrant seeds that can be difficult to procure and maintain in a viable and quiescent (non-germinating) state for the periods required to implement cryopreservation protocols. Vegetative materials are the preferred choice when preserving clonal and elite germplasms for assisting in plant breeding programmes.

Table 2 Ornamental plant species cryopreserved using various encapsulation techniques

Species	Explants cryopreserved	Method used	**References**
Begonia x erythrophylla	Adventitious shoots	Encapsulation-dehydration	Burritt (2008)
Camellia sinensis	Embryonic axis	Encapsulation-dehydration	Kaviani (2010)
Catharanthus roseus	Cell suspension cultures	Encapsulation-dehydration	Bachiri et al. (1995)
Cattleya labiata	Protocorms	Encapsulation-dehydration	Galdiano and Lemos (2018)
Chrysanthemum morifolium	Shoot tips	D cryo-plate	Tanaka et al. (2016)
Chrysanthemum x grandiflorum	Shoot tips	Encapsulation-dehydration	Zalewska and Kulus (2013)
Cosmos atrosanguineus	Cell suspension cultures	Encapsulation-dehydration	Wilkinson et al. (2003)
Dendrobium candidum	Protocorms	Encapsulation-vitrification	Yin and Hong (2009)
Dianthus caryophyllus	Shoot tips	Encapsulation-vitrification	Halmagyi and Deliu (2007)
Dianthus caryophyllus	Shoot tips	V cryo-plate	Sekizawa et al. (2011)
Dianthus spp.	Shoot tips	Encapsulation-dehydration	Fukai et al. (1994)
Gentian	Shoot tips	Encapsulation-vitrification	Tanaka et al. (2004)
Iris nigricans	Somatic embryos	Encapsulation-dehydration	Shibli (2000)
Juncus decipiens	Basal stem buds	V cryo-plate and D cryo-plate	Niino et al. (2013)
Lilium ledebourii	Embryonic axes	Encapsulation-vitrification	Kaviani (2011)
Lolium L	Meristem apices	Encapsulatin-dehydration	Chang et al. (2000)
Marchantia polymorpha	Gemma	V cryo-plate and D cryo-plate	Tanaka et al. (2016)
Oncidium bifolium	Protocorms	Encapsulation-dehydration	Flachsland et al. (2006)
Paeonia lactiflora	Zygotic embryos	Encapsulation-dehydration	Kim et al. (2004)
Passiflora pohlii	Root tips	V cryo-plate	Simao et al. (2018)
Pelargonium spp.	Shoot tips	Encapsulation-dehydration	Grapin et al. (2003)
Rhododendron simsii	Shoot tips	Encapsulation-dehydration	Verleysen et al. (2005)

(continued)

Table 2 (continued)

Species	Explants cryopreserved	Method used	**References**
Rosa 'New Dawn'	Shoot tips	Encapsulation-dehydration	Pawlowska and Bach (2011)
Saintpaulia ionantha	Shoot tips	Encapsulation-vitrification	Moges et al. (2004)
Tanacetum cinerariifolium	Shoot tips	V cryo-plate	Yamamoto et al. (2011)
Zoysia	Meristem apices	Encapsulation-dehydration	Chang et al. (2000)

Somatic embryos developed from non-sexual cells are also commonly used in encapsulation cryopreservation. Somatic embryos are structurally similar to zygotic embryos having both apical and basal meristematic regions with the ability to form shoots and roots and grow into complete plants. However, unlike zygotic embryos, somatic embryos are duplicates of a single genotype and thus can be used for clonal propagation. To successfully cryopreserve somatic embryos, particular attention needs to be given to the developmental stage of the embryos, plus the age and physiological status of the donor tissue and the cell density (Rout et al. 2006).

Root cultures with highly uniform morphology and high regeneration ability are a useful model material for cryopreservation. Root culture materials can be sourced from the field, in the case of higher plants and also from in vitro grown hairy root cultures without inflicting lethal damage to the growing donor plant. Isolation of root tips is generally straightforward and they can be treated in a similar manner to shoot tips. One further advantage of root tip cryopreservation is that it guarantees genetic stability, particularly for chimeric plants as the root tips are formed from a simple structure with one histogen layer (Simao et al. 2018; Kulus and Zalewska 2014). Table 2 summarises root culture cryopreservation of selected ornamental plant species using encapsulation technique.

5.3 *Technological Advances in Encapsulation Cryopreservation*

5.3.1 Incorporation of Additives

The encapsulation matrix not only provides protection from desiccation and mechanical injury but also provides a medium in which nutrients, fungicides, pesticides, antibiotics, antioxidants and microorganisms can be incorporated. This is especially useful for embryonic axis cryopreservation (Nadarajan and Pritchard 2014; Owen et al. 2014; Malik and Chaudhury, 2006) as excision from the seed can lead to wounding stress and a burst of superoxide ($O2^{\cdot-}$) in cells (Roach et al. 2008). Such an oxidative 'burst' will eventually result in low or nil viability of the tissue. In this context, supplying free radical scavenging capability exogenously can assist tissue survival during cryopreservation. Incorporation of antioxidant(s) into

the encapsulating gel could overcome oxidative burst problems and improve survival. This has been demonstrated in cryopreservation of *Dioscorea alata* and *D. cayenensis* shoot tips, where the incorporation of exogenous melatonin in the Ca-alginate bead matrix improved survival following cryopreservation (Uchendu and Keller 2016). Hirata et al. (2002) also found that pre-culture and encapsulation of the *Vinca minor* hairy roots in the presence of abscisic acid (ABA) were effective in increasing survival rate after cooling in liquid nitrogen.

It has been reported that incorporation of activated charcoal improves the conversion rate and vigour of the encapsulated somatic embryos (Saiprasad 2001). Charcoal is claimed to 'break up' the alginate thus increasing respiration of somatic embryos, which otherwise lose vigour within a short period of storage. In addition, charcoal is also known to slow the release of nutrients from the hydrogel capsule to the growing embryo (Saiprasad 2001).

Wood et al. (2000) simultaneously cryopreserved seeds of orchid species; *Dactylorhiza fuchsii* and *Anacamptis morio* with hyphae of their mycorrhizal fungi, *Ceratobasidium cornigerum* in encapsulated alginate beads. They reported that the viability of the seeds and the fungus remained unchanged during 30 days of cryopreservation. Sommerville et al. (2008) used sucrose-infused alginate to encapsulate two Australian orchid seeds together with their mycorrhizal fungi and reported that the sucrose addition to the alginate matrix improved seedling growth, and lessened the shock of transfer to potting mix by providing an immediate food source for the fungus and hence an uninterrupted supply of nutrients for the seedlings. These results indicate opportunities for the use of simultaneous cryopreservation of encapsulated material as a conservation tool for diverse taxa.

5.3.2 Cryo-Plate and Cryo-Mesh Methods

A new cryopreservation method using aluminium cryo-plates (V cryo-plate and D cryo-plate) has been developed for clonally propagated crops by Yamamoto et al. (2011) and Niino et al. (2013). In V cryo-plate methods, pre-cultured explants are encapsulated in alginate gel on small wells of cryo-plates and treated with LS, followed by PVS2 treatment before cryopreservation. In the D cryo-plate method, following attachment onto a cryo-plate the explants are air dried under the air current of a laminar flow cabinet before cryopreservation. It was reported that these materials are easy to handle, resulting in minimal injury to explants, plus the rapid rates of cooling and warming resulted in high regrowth rates. The cryo-plate method has been applied successfully to over 20 crop species and some ornamental species as listed in Table 2.

Funnekotter et al. (2017) investigated an alternative to the cryo-plate protocol where an aluminium mesh (cryo-mesh) was used instead of the cryo-plate with fixed well sizes. Cryo-plates require specific and consistent production of wells or shoot tips may not be able to adhere. Cryo-mesh overcomes this by allowing the alginate to act as glue between the mesh and the shoot tips. Cryo-mesh efficacy was tested in comparison with droplet-vitrification during cryopreservation of the West Australian species *Anigozanthos viridis* (Funnekotter et al. 2017). No significant difference in

post-cryogenic generation was observed between the two protocols: 78% for droplet-vitrification and 83% for cryo-mesh. Cryo-mesh, however, was claimed to reduce both the manufacturing precision required (compared to cryo-plates) and the operator skill required in comparison with the droplet-vitrification protocol.

5.3.3 Water Thermal Analysis

Understanding the physical properties of water in plant tissues being cryopreserved is pivotal as water-solute molecular properties provide information about ice formation potential, glass transition and vitrified state of the samples (Nadarajan and Pritchard 2014). To ensure the survival of cryopreserved materials, it is critical to achieve a stable glass transition and to avoid the formation of ice. Thermal analysis using a differential scanning calorimetry (DSC) is used to optimise vitrification-based cryoprotection strategies: this integration of fundamental and applied approaches advances the development of storage protocols (Nadarajan et al. 2008; Nadarajan and Pritchard 2014) and has been used to assist in cryopreservation protocol development of many species (Dereuddre and Kaminski 1992; Block 2003; Zámečník and Šesták 2010). Thermal analysis is relatively straightforward where a good thermal contact can be easily achieved by placing the samples directly inside the DSC crucibles for analysis. However, for encapsulated materials, thermal analysis data need to be interpreted carefully as the alginate matrix that covers the tissue could give a false indication of water status inside the tissue. Use of empty alginate beads as a reference or to establish a baseline will enable thermal events related to tissue only to be distinguished precisely (Block 2003). A study by Umair (2011) showed that thermal behaviour of encapsulated zygotic embryos of *Cycas revoluta* varied greatly from non-encapsulated embryos: encapsulated embryos needed longer drying periods to achieve a vitrified state or glass transition compared to non-encapsulated embryos (Fig. 2). Nadarajan et al. (2008) utilised DSC thermal analysis to reveal the capability of different and complex cryoprotectant combinations to achieve stable glasses on cooling and rewarming for encapsulation-vitrification cryopreservation of shoot tips of *Parkia speciosa*. Gamez-Pastrana and Gonzalz-Arnao (2011) used empty alginate beads as a model to investigate water thermal behaviour at every successive step in encapsulation-dehydration and encapsulation-vitrification cryopreservation protocols. They noted that during encapsulation-dehydration, freezable water was significantly reduced during the pre-culture step and when pre-culture was combined with desiccation, glass transition was observed. They also found that in the encapsulation-vitrification procedure, loading solution treatment reduced freezable water and PVS2 was found to be more effective in removing freezable water from samples compared to PVS3.

In summary, technology is developing where artificial seed production in the form of encapsulated propagules could be more effective and efficient in plant propagation, in reducing stress as well as prolonging storage life of the encapsulated propagules either for medium- or long-term.

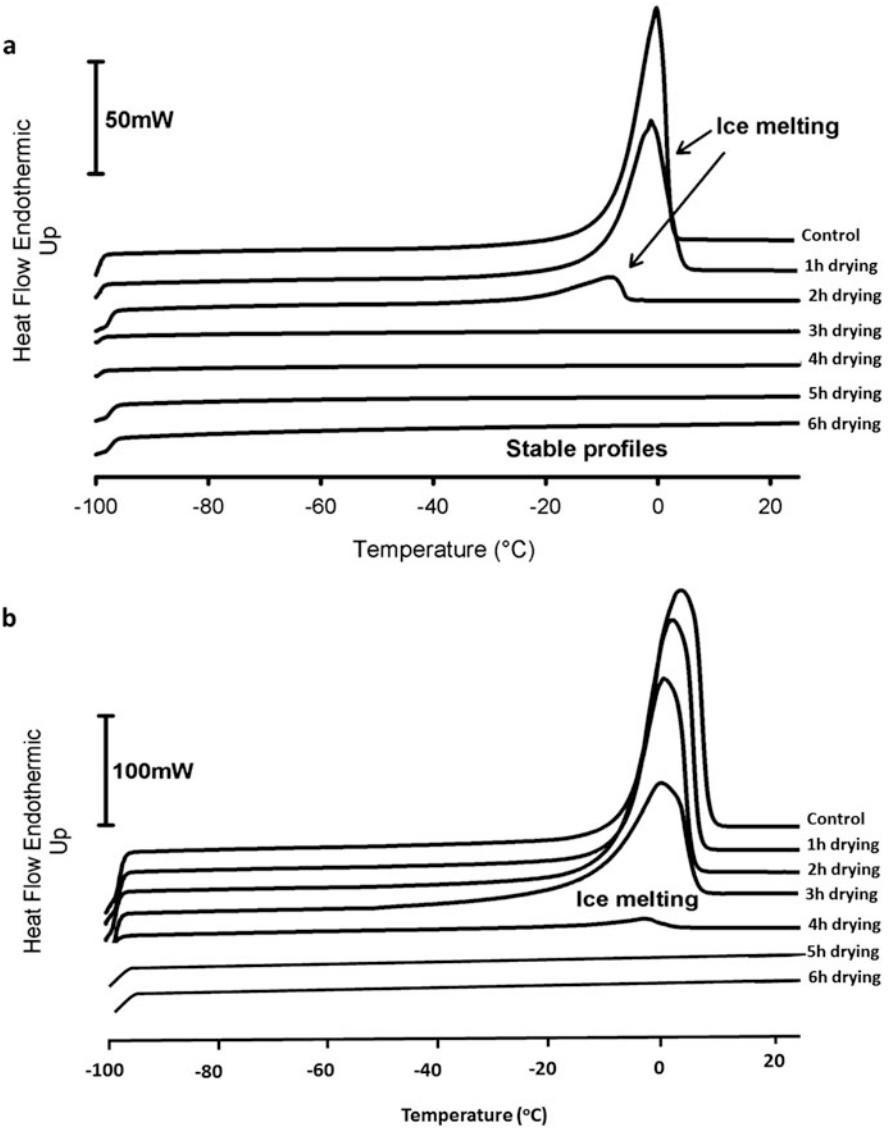

Fig. 2 Differential scanning calorimetry warming thermograms of *Cycas revoluta* non-encapsulated (**a**) and encapsulated (**b**) zygotic embryos following desiccation under the air current of a laminar flow cabinet for up to 6 h. Non-encapsulated embryos showed ice melts up to 2 h of drying and then followed by stable thermal profiles. Encapsulated embryos showed ice melt up to 4 h drying before a stable profile is obtained. Samples were warmed from −100 °C to 25 °C at 10 °C min^{-1} (modified from Umair 2011)

6 Conclusions

This review summarises the need for ornamental plant germplasm conservation and how synthetic seed technology could complement other conservation strategies to conserve this valuable germplasm. Encapsulated or synseeds have advantages including easy handling, storage, transferring, preservation of elite or endemic ornamental plant species for cryopreservation, exchange of non-contaminated plant materials among laboratories, gene banks and industries. Synthetic seed technology allows medium-term germplasm conservation under in vitro condition at low temperature (below 0 °C) and reduces the needs for frequent sub-culturing. Cryopreservation technology for artificial seeds provides the opportunity for long-term conservation, which can be implemented for genotypes of a large number of species in gene banks and large-scale germplasm conservation programmes. Anticipated advances in artificial seed production technology in combination with advances in tissue culture and cryobiology technology will further assist the future conservation of ornamental plant germplasm in a more efficient manner.

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Synthetic Seed Production of Flower Bulbs

Metin Kocak, Basar Sevindik, Tolga Izgu, Mehmet Tutuncu, and Yesim Yalcın Mendi

Abstract Flower bulbs are perennial or annual plants with underground structures such as bulb, corn, tuber, and rhizomes. These plants have economic value especially in ornamental plant sector as cut flower, potted flower, and outdoor plants. Most of these plants have garish flower, and many of them are monocotyledon. *Cyclamen*, *Tulipa*, *Lilium*, *Narcissus*, *Gladiolus*, *Hyacinthus*, *Crocus*, *Iris*, *Allium*, *Alstroemeria*, *Anemone*, *Orchis*, *Rhododendron*, *Freesia*, *Hippeastrum*, *Muscari*, *Ornithogalum*, *Ranunculus*, and *Zantedeschia* are the most important geophytes that are commercially used in the world. These plants can be propagated using conventional and tissue culture techniques. Synthetic seed production is one of these techniques. Synthetic seed, namely, artificial seed, is described as artificially encapsulated plant tissues and somatic embryos with alginate hydrogel. Synthetic seed technology has significant effect on the conservation of the plant tissues and sustainability of the plants. Recently, conservation of the plant species studies significantly increased, and artificial seed method was used as the most common process to conserve important species. In this chapter, oldest and newest synthetic seed production researches were discussed and presented chronologically.

Keywords Synthetic seed · Encapsulation · Flower bulbs · Propagule · Protocorm-like bodies

M. Kocak
Faculty of Agriculture, Department of Agricultural Biotechnology, Van Yüzüncü Yil University, Bardakçı, Van, Turkey

B. Sevindik
Vocational High School, Izmir Demokrasi University, İzmir, Turkey

T. Izgu
Faculty of Agriculture, Department of Horticulture, University of Ege, Bornova, İzmir, Turkey

M. Tutuncu
Faculty of Agriculture, Department of Horticulture, Ondokuz Mayıs University, Atakum, Samsun, Turkey

Y. Y. Mendi (✉)
Faculty of Agriculture, Department of Horticulture, University of Çukurova, Sarıçam, Adana, Turkey

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1 Introduction

Flower bulbs (Geophytes) including underground structures like bulb, corm, tuber, and rhizomes (Dafni et al. 1981) have economic value due to their usage in different areas as food production, medicinal, and aromatic and landscape (Zaidi et al. 2000; Çığ and Başdoğan 2015). Storage organs include vitally important nutrients (water, proteins, carbohydrate, minerals, etc.) that are used in long dormancy period of the plant. Most of the wild geophytes are distributed across the temperate, tropic, and subtropic zones of the world. These plants are used as cut flower, potted flower, and gardening in ornamental plant sector (Kamenetsky and Okubo 2012). According to Kazaz (2016), flower bulbs were cultivated in 30.066 ha all over the world (Kazaz 2016; Karagüzel et al. 2007; AIPH and Union fleurs 2010-2016). The value of this production is referred to more than 708.5 million € (Kazaz 2016; AIPH and Union fleurs 2010–2016).

In conventional production, ornamental geophytes are generally propagated vegetatively, and they have low production rate (Ziv and Lilien-Kipnis 2000). Besides, some problems and limitations are present in conventional production of ornamental geophytes. In vitro techniques can be used victoriously to overcome these problems and limitations. Therefore, propagation and conservation of ornamental geophytes under in vitro condition become crucial (Ziv and Lilien-Kipnis 2000). In vitro plant propagation techniques have some advantages: production of a numerous new mature cultivars in a short span of time, cloning endangered species, propagation of large amount of clone plants and some elite plants that have desirable characteristics, plant production in the absence of seeds, and production of genetically modified plants and pest disease pathogen-free plants (Yildiz 2012). In tissue culture studies of the geophytes, corms, bulbs, rhizomes, cutting, anthers, ovules, leaves, and stems are commonly used as explants (Eeckhaut et al. 2018)

Nowadays, many tissue culture applications are used for propagation and conservation of geophytes. Synthetic seed production is one of the significant tissue culture techniques. Synthetic seed that is also known as artificial seed, manufactured seed, or synseed was firstly described by Murashige (Murashige 1977) as “an encapsulated single somatic embryo.” Additionally axillary shoots, apical shoot tips, buds or stem, root segments, embryogenic calli, and protocorms or protocorm-like bodies also have potential usage for synthetic seed production (Zulkarnain et al. 2015; Siong et al. 2012; Vdovitchenko and Kuzovkina 2011). Consequently, synthetic seeds are determined as artificially encapsulated plant tissues such as shoot buds, cell aggregates, or any other tissue and somatic embryos, which can be used for sowing as a seed and that possess the regeneration ability under in vitro or ex vitro conditions and that retain this potential also after storage (Magray et al. 2017). Although several propagules have potential as plant material to produce synthetic seed, usually somatic embryos and axillary shoot buds have been preferred as explant for synthetic seed studies (Zulkarnain et al. 2015; Siong et al. 2012). For cost-efficient and highly productive proliferation method, somatic embryo coated with a synthetic layer was indicated as suitable plant material (Redenbaugh 1990; Gantait et al. 2017).

It is considered that synthetic seed is an imitated natural seed and comprises of embryo and one or more synthetic layers (capsule) (Murashige 1977; Baskaran et al. 2015; Cangahuala-Inocente et al. 2007; Maqsood et al. 2015). The capsule consists of gel agent and additional materials such as nutrients, growth regulators, anti-pathogens, bio-controllers, and bio-fertilizers (Rihan et al. 2017; Cartes et al. 2009), and it participates in preservation of plant material during handling and provides germination of synthetic seed like a real seed and formation of plantlet under proper circumstances (Zulkarnain et al. 2015).

Synthetic seed technology allows for conservation and large-scale micropropagation of special rare hybrids, elite genotypes, sterile unsteady genotypes, and genetically modified plants (Gantait et al. 2015). In addition, it provides forceful option for propagation of commercially valuable crop which cannot produce seeds irrespective of season and climatic conditions (Taha et al. 2013; Grzegorczyk and Wysokińska 2011). Synthetic seed technology has developed correlatively with improvement of somatic embryogenesis and culture system design, providing compatibility between somatic embryogenesis and capsulation (Onishi et al. 1994; Çölgeçen and Toker 2006). However, application of this technology is faced with some restrictions: insufficient viable propagule production for synthetic seed production, abnormal and asynchronous development of somatic embryos, unsuitable maturation of somatic embryos, lack of dormancy and stress tolerance in synthetic seeds to be stored, and weak conversion of propagules into plantlets (Ara et al. 2000).

2 Types of Synthetic Seed

Synthetic seeds are mainly separated into two groups as encapsulated desiccated synthetic seeds and encapsulated hydrated synthetic seeds.

2.1 Desiccated Synthetic Seeds

The desiccated synthetic seeds are produced by desiccation of somatic embryos either naked or encapsulated in polyethylene glycol. Both slow and rapid desiccations can be carried out based on certain conditions. Although in rapid desiccation, synthetic seeds can be desiccated in unsealed petri dishes overnight, slow desiccation of synthetic seeds takes 1 or 2 weeks' time. To produce desiccated synthetic seeds from a plant species, somatic embryos of this plant species must be tolerant to desiccation. Seyring and Hohe (2005) tested the tolerance of somatic embryos of *Cyclamen persicum* Mill. Torpedo-shaped somatic embryos (sized 700–1000 μm) showed the best germinability after desiccation. The germination rate of desiccated somatic embryos reached 28% with supplement of 75 g l^{-1} polyethylene glycol (PEG 4000) and 10 mg l^{-1} abscisic acid in maturation medium. Khor et al. (1998)

encapsulated desiccated protocorms and seeds of *Spathoglottis plicata* with alginate–chitosan or alginate–gelatin. About 54% of large protocorms and 40% of seeds could tolerate a 6-h desiccation. All the protocorms and seeds encapsulated with alginate–chitosan or alginate–gelatin after desiccation were able to survive.

2.2 Hydrated Synthetic Seeds

Hydrated synthetic seeds are produced by encapsulating somatic embryos or other propagules in hydrogel capsules. Hydrogel encapsulation was developed by Redenbaugh et al. (1984) by encapsulation of individual somatic embryos of alfalfa (*Medicago sativa*), and since their study, this encapsulation approach has been the most preferred technique (Sharma et al. 2013; Ara et al. 2000; Rai et al. 2009). In comparison with desiccated synthetic seeds, hydrated synthetic seeds can be produced by encapsulating somatic embryos that are recalcitrant and sensitive to desiccation. Despite the presence of many gel agents like potassium alginate, agar, gelrite, and sodium pectate, calcium alginate was suggested as more suitable than the other gel agents (Nongdam 2016; Redenbaugh et al. 1987). To obtain artificial seed drop beads, different chemicals combined with Na-alginate were used. In most of the studies, explants covered by Na-alginate were dropped into the $CaCl_2$ to obtain drop beads. However, Haque and Ghosh (2016) used $CaNO_3$ to obtain drop beads for *Ledebouria revoluta* bulb explant.

3 Propagule Types

Protocorm, protocorm-like body, somatic embryo, bulb, seed, shoot bud, and shoot tip have been used as propagule for production of synthetic seeds of flower bulbs. In literature, although there have been many reports for production of synthetic seed in orchids, the reports for other plant groups of flower bulbs have been restricted.

Protocorm and Protocorm-Like Bodies (PLBs) Protocorm-like bodies (PLBs) provide an efficient propagating technique for orchids; therefore, they have been usually preferred to produce synthetic seed in orchids. Protocorm-like bodies are used as an explant for both artificial seed and cryopreservation researches. In the artificial seed studies of some orchid species, PLBs have high regeneration response (Datta et al. 1999; Jitsopakul et al. 2008; Khoddamzadeh et al. 2011; Mohanty et al. 2013; Bhattacharyya et al. 2018).

Somatic Embryos The other propagule used for synthetic seed production in flower bulbs is somatic embryo. Somatic embryos are the most preferred propagule in synthetic seed technology, but only a few studies have been reported on synthetic seed production with somatic embryos in flower bulbs. Winkelmann et al. (2004)

used somatic embryos that were globular stage to produce synthetic seeds in *Cyclamen persicum* from ovule cultures. They produced somatic embryos in a liquid culture system and examined two encapsulation techniques: conventional alginate beads and alginate hollow beads. In both encapsulation systems, high level of germination was observed. In the other study reporting synthetic seed production in *Cyclamen persicum*, Seyring and Hohe (2005) tested desiccation tolerance of torpedo-stage embryos. Haque and Ghosh (2014) developed somatic embryogenesis protocol for *Drimiopsis kirkii* from leaf explant and encapsulated somatic embryos which are produced via their protocol. Encapsulated somatic embryos with 1% sodium alginate showed the highest germination rate (93.3%). Haque and Ghosh (2016) encapsulated somatic embryos at globular to elongated stage of *Ledebouria revoluta* with different concentrations of sodium alginate (1.5, 3.0, and 4.5%). In the present study, 57.8% of encapsulated somatic embryos germinated.

Bulb The bulbs obtained from the cultures of rhizome segments of *Ipsea malabarica* were encapsulated with sodium alginate by Martin (2003). The highest conversion rate was reported as 100% in this study. Yücesan et al. (2014) also used bulblets as propagule to produce synthetic seed in grape hyacinths (*Muscari armeniacum*). They developed a somatic embryogenesis protocol and encapsulated bulblets that regenerated via their protocol. High incidence of synthetic seeds (95%) showed germination in this study.

Shoot Tip Gantait and Sinniah (2013) developed a short-term storage protocol for synthetic seeds of orchid hybrid *Aranda* Wan Chark Kuan 'Blue' × *Vanda coerulea* Grifft. ex. Lindl. Synthetic seeds were prepared by encapsulating shoot tips (3–4 mm) with 3% sodium alginate, and almost all of the encapsulated shoot tips (99.6%) showed germination. Another study including encapsulation of shoot tips was reported by Baskaran et al. (2017) in *Urginea altissima* (L.f.) Baker. In this study, shoot tips were obtained from leaves and longitudinal thin cell layer leaf culture of *Urginea altissima* by using semisolid and liquid culture systems. Then shoot tips were encapsulated with MS medium plus 3% (w/v) sodium alginate. The synthetic seeds showed a max 91% germination.

Shoot Bud To produce synthetic seed of *Curculigo orchioides*, Nagesh et al. (2009) preferred shoot bud as propagule. Different concentrations of sodium alginate (1.5, 2.0, and 2.5%) were used for encapsulation of 5–6.0-mm-sized shoot buds. The highest germination rate of synthetic seeds was reported as 68.8%.

Seed Khor et al. (1998) desiccated seeds of *Spathoglottis plicata* and encapsulated with alginate–chitosan or alginate–gelatin. A hundred percent of desiccated synthetic seeds encapsulated with both of alginate–chitosan or alginate–gelatin was able to germinate.

4 Hydrogel Encapsulation Techniques

In synthetic seed production, various encapsulation techniques such as single-layered encapsulation, double-layered encapsulation, and hollow beads have been utilized. In literature, single-layered encapsulation and hollow beads have been reported for synthetic seed production of flower bulbs.

4.1 Single-Layered Encapsulation

Single-layered encapsulation technique is the most ordinary and the most preferred hydrogel encapsulation method. This encapsulation technique is applied by coating of propagules with a hydrogel agent, generally sodium alginate or calcium alginate, and dropping them to calcium chloride or calcium nitrate solution to solidify the outer surface of the synthetic layer. In this technique, seeds, somatic embryos, protocorm-like bodies, bulb segments, and bulbs were used as explants (Datta et al. 1999; Kaviani 2010; Haque and Ghosh 2016; Bhattacharyya et al. 2018). Bhattacharyya et al. (2018) indicated that synthetic seeds of *Ansellia africana* protocorm-like bodies obtained from the nodal segments were regenerated with the response of 88.21%. They used 3% alginate and 100 ml $CaCl_2$ for encapsulation. In another study, Haque and Ghosh (2016) encapsulated the somatic embryos of the *Ledebouria revolute* with different concentrations (1.5, 3, 4.5%) of the Na-alginate, and they used $Ca(NO_3)$ to form the drop beads. Researchers reported that the artificial seeds were regenerated after 4 months in 15 °C cultivation with the response of 57%.

4.2 Hollow Beads

Because of mostly situating propagules near the surface of bead, traditional single-layered synthetic seeds are not sufficient as natural seeds to preserve the propagule. Hollow beads have been considered to mimic natural seeds better than traditional single-layered synthetic seeds. Although hollow beads have some advantages compared with natural seeds, naked somatic embryos, and encapsulated somatic embryos such as complete protection, easy handling, and rapid clonal propagation, the application of hollow bead technique is labor-intensive and costlier (Sharma et al. 2013; Patel et al. 2000). Winkelmann et al. (2004) examined alginate hollow beads and classic alginate encapsulation techniques by encapsulating globular-stage somatic embryos of *Cyclamen persicum*. They informed that the final germination rate (97%) of classic encapsulated synthetic seeds was higher than germination rate (7%) of hollow beads. Although somatic embryos were located centrally in hollow bead, they were not coated totally in alginate beads. In hollow beads, different growth stages such as cotyledons, root, and tuberization occurred in the capsule. But in alginate beads, somatic embryos enlarged and spread out of the capsule in the early stages of germination.

5 Storage of Synthetic Seeds

Synthetic seeds are not only used for plant propagation but also used for plant genetic germplasm preservation. Synthetic seed technology has brought new perspectives to plant germplasm transportation, characterization, preservation, and genetic resource management (Ahmed et al. 2014). Synthetic seeds can be stored for short term and long term.

5.1 Short-Term Germplasm Conservation

The success of germplasm conservation of synthetic seed is directly associated with storage temperature. Short-term germplasm conservation has been performed at different temperatures (4–26 °C) in flower bulbs. Some researchers indicated that conservation of synthetic seeds at 4 °C has been the optimal storage temperature (Saiprasad and Polisetty 2003; Nagesh et al. 2009; Pradhan et al. 2016; Haque and Ghosh 2017). Pradhan et al. (2016) produced synthetic seeds of *Cymbidium aloifolium* by encapsulating protocorms with calcium alginate. They cultured synthetic seeds in liquid culture containing MS or Kn C media supplemented with 0.5 mg/l 6-benzyl aminopurine (BAP) and 0.5 mg/l α-naphthalene acetic acid (NAA) at 4 °C and room temperatures (RT, 21 °C ± 2 °C) during 90 days. The synthetic seeds stored at 4 °C had higher germination ability than stored at room temperature in both of MS Kn C media. Haque and Ghosh (2017) encapsulated PLBs of *Spathoglottis plicata* with sodium alginate and stored these synthetic seeds at three different temperatures (4, 15, and 24 °C). Although synthetic seeds stored at room temperature (24 °C) showed the best germination rate in the 30th day of storage, synthetic seeds stored at 4 °C showed the best germinability with 66.7% after 120 days of storage. Although some researchers indicated that storage at 4 °C is the most suitable for synthetic seed conservation, others reported that storage at higher temperatures is the best one (Gantait et al. 2012; Gantait and Sinniah 2013; Mohanty and Das 2013; Haque and Ghosh 2014; Mahendran 2014; Haque and Ghosh 2016; Baskaran et al. 2017; Bhattacharyya et al. 2018). Haque and Ghosh (2016) stored encapsulated somatic embryos of *Ledebouria revoluta* at different temperatures (4, 15, and 24 °C). After 180 days of storage, synthetic seeds stored at 15 °C gave the best result with 11.1% germination rate, while synthetic seeds stored at 4 and 24 °C lost their germinability. Bhattacharyya et al. (2018) developed an efficient short-term storage system for synthetic seed of *Ansellia africana*. The synthetic seeds which were produced by encapsulating PLBs with 3% sodium alginate were stored at 4, 8, and 25 °C during 90 days. At the end of the storage, the best germination rate was obtained from storage at 4 °C.

5.2 *Long-Term Germplasm Conservation (Cryopreservation)*

Cryopreservation is an efficient technique for long-term conservation of plant germplasm. This technique relies on storage of tissues at ultralow temperatures in liquid nitrogen (−196 °C) or rarely in vapor phase (−150 °C). Storage of tissues for long term in this method can be achieved because of pausing metabolic activities and cell division in ultralow temperatures. Cryopreservation provides some advantages such as cost-efficient in vitro cultures, minimum space requirement, decrease of contamination, and somaclonal variation risk (Kulus and Zalewska 2014). Da Silva (2012) tested both short-term and long-term (cryopreservation) conservation of synthetic seeds obtained from PLBs of hybrid Twilight Moon "Day Light." According to the results of this experiment, it was found that short-term conservation of *Cymbidium* was applicable, while long-term (cryopreservation) conservation was not efficient.

6 Genetic Fidelity of Synthetic Seeds

Somaclonal variation is a common problem in tissue culture. DNA marker systems provide an efficient tool to determine genetic stability of in vitro-derived plants. Because of being cost-effective, faster, simpler, and not to need DNA sequence information, the random amplified polymorphic DNA (RAPD) marker system has been used to identify somaclonal variations of synthetic seed-derived plants (Weising et al. 2005, Rizkalla et al. 2012, Nybom et al. 2014, Haque and Ghosh 2016). Mohanty and Das (2013) encapsulated PLBs of *Dendrobium densiflorum* Lindl. Ex Wall to produce synthetic seed and stored them up for 90 days. To test genetic fidelity of regenerated plants, 10 RAPD primers were used, and a total of 39 scorable bands were detected with 3.9 bands per primer. In the present study, no variation was found between regenerated plants from synthetic seeds and the parental plant. Haque and Ghosh (2016) used RAPD marker system to compare genetic fidelity among mother plant and synthetic seed-derived plants of *Ledebouria revoluta*. A total of 72 bands were obtained with 17 RAPD primers, and the results revealed that there was no variation within or between the regenerated plants and mother plant. Other two studies to check genetic fidelity between synthetic seed-derived plants and mother plant were reported by Haque and Ghosh (2014) in *Drimiopsis kirkii* and Baker and Mishra et al. (2011) in *Picrorhiza kurroa*. In both of these studies, regenerated plants and parental plants were found stabile genetically. Another molecular marker system used to assess genetic variation in synthetic seed technology is inter simple sequence repeats (ISSR). Gantait and Sinniah (2013) carried out PCR amplification with ISSR primers to assess genetic stability of synthetic seed-derived plants obtained from alginate-encapsulated shoot tips of monopodial orchid hybrid *Aranda* Wan Chark Kuan 'Blue' x *Vanda coerulea* Grifft. ex. Lindl. A total of 51 monomorphic bands were produced with 9 ISSR primers,

and results demonstrated genetic uniformity between clones. Bhattacharyya et al. (2018) preferred IRAP (inter-retrotransposon amplified polymorphism) and SCoT (start codon targeted) markers that target genes to evaluate genetic fidelity of regenerated *Ansellia africana* (leopard orchid) via synthetic seeds. High level of genetic uniformity was found between in vitro-derived plants.

The concept of synthetic seed production in flower bulbs is given schematically in Fig. 1 that presents applied methods in literature, and synthetic seed production process is presented in *Cyclamen* sp. in Fig. 2. Also, studies and reports on synthetic seed production of flower bulbs are given chronologically in Table 1.

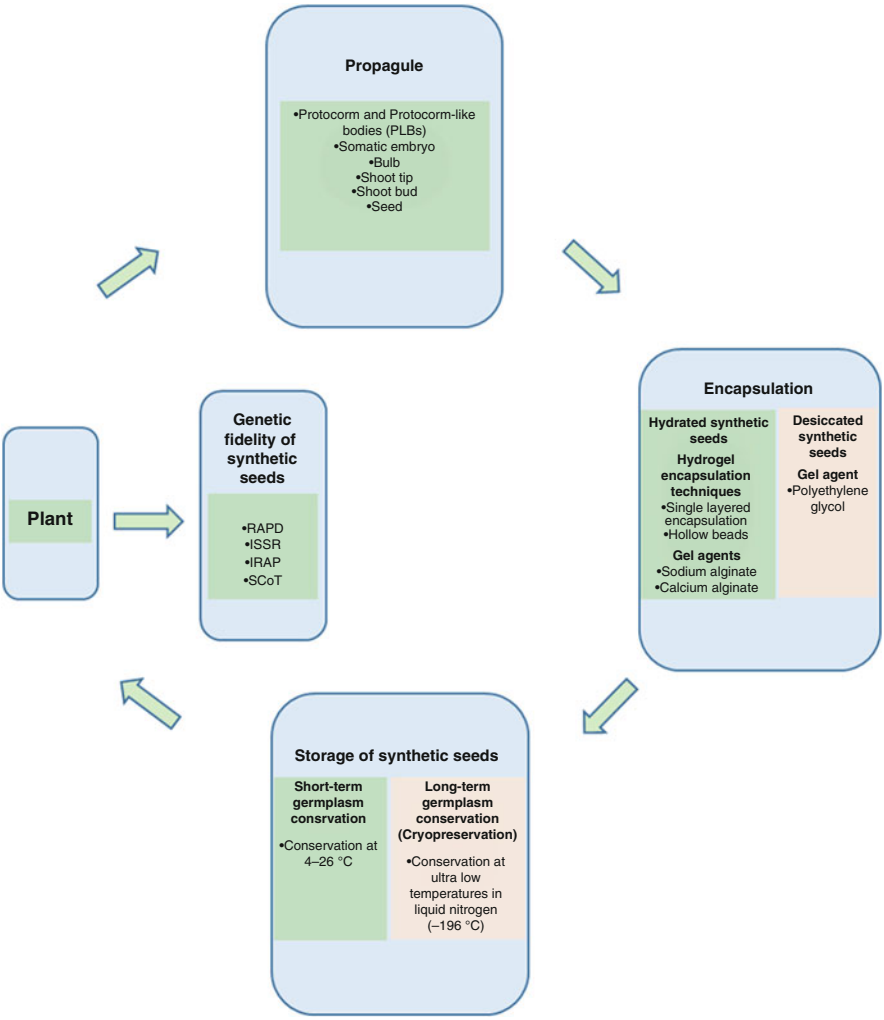

Fig. 1 The concept of synthetic seed production in flower bulbs

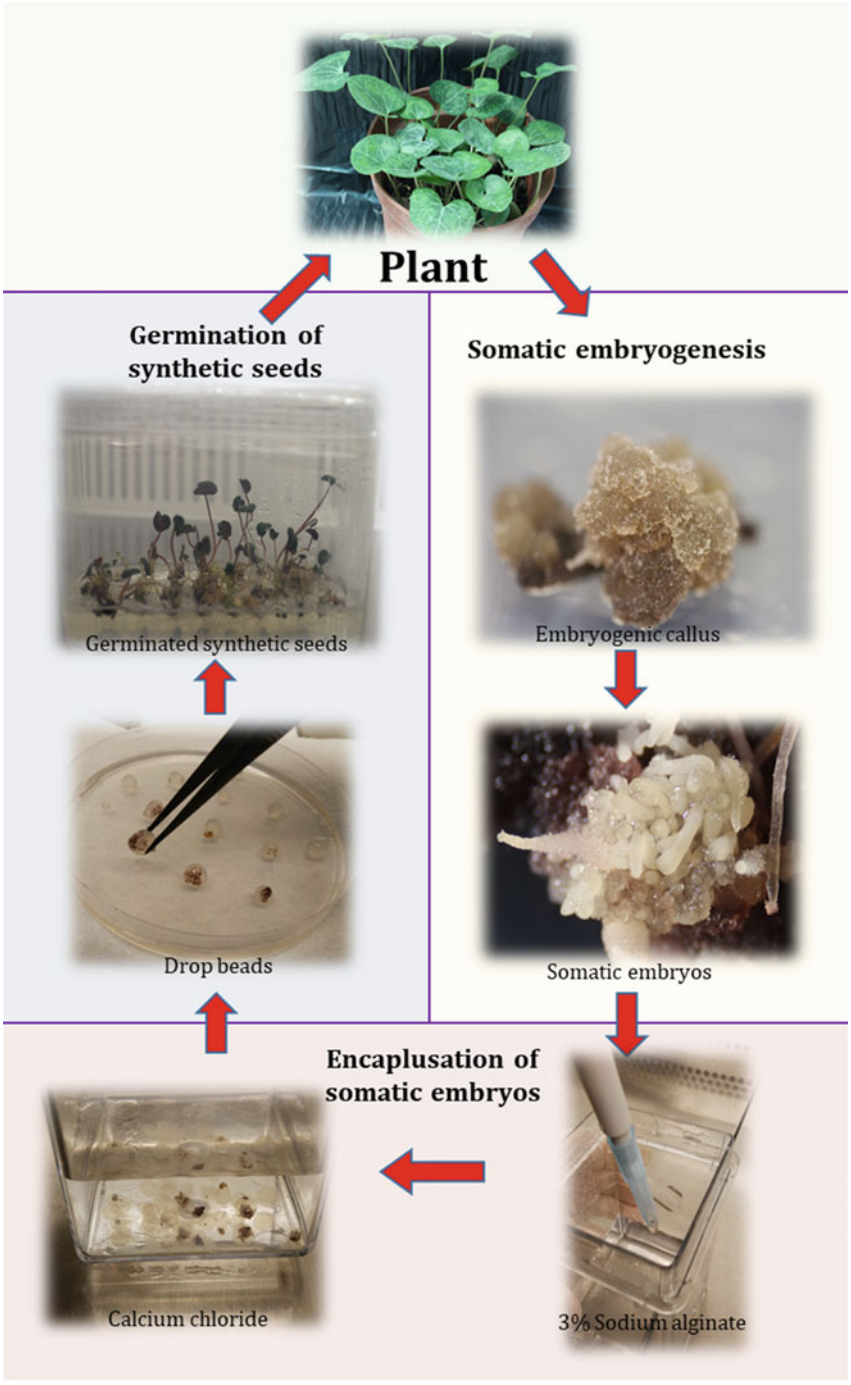

Fig. 2 Synthetic seed production at *Cyclamen* sp. by encapsulating somatic embryos

Table 1 Researches on synthetic seed production of flower bulbs

Plant (species)	Explant type	Type of synthetic seed/ gel agent	Result (survival rates)	References
Spathoglottis plicata	Protocorm and seed	Desiccated synthetic seed Alginate–chitosan, alginate–gelatin, sodium alginate	2.3–100% Depending on encapsulation agents, explant type and size, desiccation, and combination of these factors	Khor et al. (1998)
Geodorum densiflorum (Lam) Schltr.	Protocorm-like bodies	Hydrated synthetic seed Sodium alginate	20.4–88% Depending on storage time and storage temperature	Datta et al. (1999)
Dendrobium 'SONIA' *Oncidium* 'GOWER RAMSAY' *Cattleya* LEOPOLDII	Protocorm-like bodies	Hydrated synthetic seed Sodium alginate	Depending on storage temperature and period: 12–100% for *Dendrobium*, 36–100% (4 °C) for *Oncidium*, 16–100% (4 °C) for *Cattleya*	Saiprasad and Polisetty (2003)
Ipsea malabarıca (Reıchb. f.) J. D. Hook.	Bulb	Hydrated synthetic seed Sodium alginate	100%	Martin (2003)
Cyclamen persicum	Somatic embryo	Hydrated synthetic seed Alginate beads, hollow beads	97% for alginate beads, 71% for hallow beads	Winkelmann et al. (2004)
Cyclamen persicum Mill.	Somatic embryo	Desiccated synthetic seed Polyethylene glycol	28%	Seyring and Hohe (2005)
Curculigo orchioides	Shoot bud	Hydrated synthetic seed Sodium alginate	28–68.3% for sodium alginate concentration, 9.5–68.8% for CaCl2 exposure time, 0.62–64.5% for storage temperature and period	Nagesh et al. (2009)
Coelogyne breviscapa Lindl.	Protocorm-like bodies	Hydrated synthetic seed Sodium alginate	33.6–91% Depending on storage time	Mohanraj et al. (2009)
Vanda coerulea Grifft. ex. Lindl.	Protocorm-like bodies	Hydrated synthetic seed Sodium alginate	23%–94.9% Depending on storage time, concentration of sodium alginate, and calcium chloride	Sarmah et al. (2010)

(continued)

Table 1 (continued)

Plant (species)	Explant type	Type of synthetic seed/ gel agent	Result (survival rates)	References
Flickingeria nodosa (Dalz.) Seidenf	Protocorm-like bodies	Hydrated synthetic seed Sodium alginate	6.5–95% Depending on storage time	Nagananda et al. (2011)
Picrorhiza kurroa	Microshoots	Hydrated synthetic seed Sodium alginate	Max 89.33%	Janhvi Mishra et al. (2011)
Cymbidium (hybrid)	Protocorm-like bodies	Hydrated synthetic seed Sodium alginate	6–100% Depending on storage time and method	Teixeira da Silva (2012)
Aranda Wan Chark Kuan 'Blue' x *Vanda coerulea* Grifft. ex. Lindl.	Protocorm-like bodies	Hydrated synthetic seed Calcium alginate	69.5–96.4% Depending on culture media	Gantait et al. (2012)
Aranda Wan Chark Kuan 'Blue' x *Vanda coerulea* Grifft. ex. Lindl.	Shoot tips	Hydrated synthetic seed Sodium alginate	Max 99.6% depending on MS strength	Gantait and Sinniah (2013)
Dendrobium nobile Lindl.	Protocorm-like bodies	Hydrated synthetic seed Sodium alginate	54.13–78.20% Depending on sucrose, mannitol, and different concentrations of them	Mohanty et al. (2013)
Dendrobium densiflorum Lindl. Ex Wall	Protocorm-like bodies	Hydrated synthetic seed Calcium alginate Sodium alginate	79.1–100% Depending on culture media	Mohanty and Das (2013)
Drimiopsis kirkii Baker	Somatic embryo	Hydrated synthetic seed Sodium alginate	93.3%	Haque and Ghosh (2014)
Cymbidium aloifolium (L.) Sw.	Protocorm	Hydrated synthetic seed Sodium alginate	85%	Pradhan et al. (2014)

(continued)

Table 1 (continued)

Plant (species)	Explant type	Type of synthetic seed/ gel agent	Result (survival rates)	References
Dendrobium white fairy orchid	Protocorm-like bodies	Hydrated synthetic seed Sodium alginate	80%	Siew et al. (2014)
Cymbidium bicolor Lindl.	Protocorm	Hydrated synthetic seed Sodium alginate	10.23–80% Depending on storage time and temperature	Mahendran (2014)
Muscari armeniacum Leichtlin ex Baker	Bulblet	Hydrated synthetic seed Sodium alginate	95%	Yücesan et al. (2014)
Spathoglottis plicata Blume	Protocorm-like bodies	Hydrated synthetic seed Sodium alginate		Stella et al. (2015)
Serapias vomeracea (Burm. f.) Briq.	Protocorm-like bodies	Hydrated synthetic seed Sodium alginate	60–100% Depending on germination media (in vitro or soil)	Bektaş and Sökmen (2016)
Ledebouria revoluta	Somatic embryo	Hydrated synthetic seed Calcium alginate	57.8%	Haque and Ghosh (2016)
Cymbidium aloifolium (L.) Sw.	Protocorm	Hydrated synthetic seed Calcium alginate	10–83% Depending on storage time, storage temperature, and culture media	Pradhan et al. (2016)
Renanthera imschootiana Rolfe	Protocorm-like bodies	Hydrated synthetic seed Sodium alginate	Max 100%	Gupta (2016)
Urginea altissima (L.f.) Baker	Shoot tips	Hydrated synthetic seed Sodium alginate	Max 91%	Baskaran et al. (2017)
Spathoglottis plicata Blume	PLB	Hydrated synthetic seed Sodium alginate	86.6%	Haque and Ghosh (2017)
Ansellia africana (leopard orchid)	Protocorm-like bodies	Hydrated synthetic seed Sodium alginate	88.21%	Bhattacharyya et al. (2018)

7 Conclusions

Ornamental geophytes have wide usage as cut flower, garden flower, and pot plant at homes, in gardens, and to many different places due to their aesthetic properties. Synthetic seed researches increased after the somatic embryogenesis studies evolve. The most important properties of the synthetic seed are short-time regeneration, sustainability of the clonal identity, seasonal independence for seed production, easy acclimatization, and cost minimization of the ornamental plant production. Hydrogel encapsulation is a common technique for synthetic seed production, and alginate and $CaCl_2$ are the commonly used chemicals to encapsulate the plant materials. Additionally, synthetic seeds can be used for cryopreservation studies, and this technique provides advantages for conserving the endangered plant species. Therefore, synthetic seed methods have been used for a long time which have different purposes in the plant biotechnology studies. In this chapter, synthetic seed production was presented, and the reports about the different usage of the synthetic seeds were detailed.

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Applications of Synthetic Seed Technology for Propagation, Storage, and Conservation of Orchid Germplasms

Saikat Gantait and Monisha Mitra

Abstract A synthetic seed is defined as an artificially encapsulated somatic embryo (protocorm-like bodies in case of orchids) or meristematic tissue (shoot tips, nodal segments, corm, and bulb) that portrays the role of a seed and has the proficiency of germinating into complete plantlets. Synthetic seed technology is an optimistic approach to preserve and clonally propagate different species of orchids that can be endangered or possess high esthetic value or in respect to economical and medicinal prominence which is covered extensively in this chapter. It aids as the most conventional and genuine means for generating plant propagules under in vivo conditions. Under ex vitro conditions, there is positive enhancement of the large-scale production of orchid species by bypassing the process of acclimatization. Synthetic seed technology positively implements the germplasm exchange through short-term storage. Cryopreservation is a constructive method for long-term germplasm conservation of plant propagules at ultralow temperature. Molecular marker technology is now an upcoming advanced practice to sample the germplasm systematically. Minimal progress has been made in the field of synthetic seed technology; there are still many challenges to make the technique more practical and feasible especially for the farmers and producers. The inadequacies are required to be surveyed extensively and solved so that this technology can be utilized in a long-term basis keeping in mind the principles of sustainable development and conservation.

Keywords Calcium chloride · Cryopreservation · Encapsulation · Encapsulation-desiccation · Encapsulation-vitrification · Germplasm storage · Protocorm-like body · Shoot tip · Sodium alginate

S. Gantait (✉)
Crop Research Unit (Genetics and Plant Breeding), Bidhan Chandra Krishi Viswavidyalaya, Mohanpur, Nadia, West Bengal, India
e-mail: saikatgantait@bckv.edu.in

M. Mitra
Department of Agricultural Biotechnology, Faculty of Agriculture, Bidhan Chandra Krishi Viswavidyalaya, Mohanpur, Nadia, West Bengal, India

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1 Introduction

Synthetic seeds can be regarded as artificial seeds that are formulated from somatic and non-zygotic embryos, thus opening up a new facet in agriculture, by means of propagules that can be derived from transgenic plants, non-seed-producing plants, recalcitrant seed-producing plants, and natural polyploid plants or induced-polyploid plants with superior and elite alleles. The earliest concept of artificial seed paves back to Haberlandt's postulate based on artificial embryo cultivation (1902). Originally, synthetic seed can be defined as "an encapsulated single somatic embryo." In a simpler version, a product that is derived through clonal means can be handled and utilized just like a real seed for sowing, storage, and transport and subsequently can be grown in ex vitro or in vitro condition further generating into a plantlet (Murashige 1978). Bapat et al. (1987) stated that in vitro propagules can be used to produce synthetic seeds apart from somatic embryos. According to Gray and Purohit (1991), the exact definition of synthetic seed is "a somatic embryo that is engineered for the practical use in commercial plant production." Henceforth, in a comprehensive context, synthetic seed can be defined as any kind of meristematic propagule or somatic embryo which is artificially encapsulated, and under ex vitro or in vitro condition, it can generate itself into a plantlet, or the seed can also be stored for future purpose (Capuano et al. 1998; Ara et al. 2000). Several researchers (Ara et al. 2000; Danso and Ford-Llyod 2003; Nhut et al. 2005; Faisal and Anis 2007; West and Preece 2009; Ahmad and Anis 2010; Ozudogru et al. 2011; Sharma et al. 2009a, b; Gantait et al. 2015) suggested that instead of somatic embryos, which are always tricky to handle, there is also a likelihood of the utilization of vegetative propagules, namely, shoot tips, protocorm, etc. which are derived from non-embryogenic origin. In addition, organogenic or embryogenic callus can also be utilized (Ara et al. 2000; Danso and Ford-Llyod 2003; Faisal and Anis 2007; Ahmad and Anis 2010). There has been immense progress in the field of synthetic seed (Sharma et al. 2013), and ample numbers of reports are available on artificial seed for orchids. In fact, synthetic seed technology in orchid biotechnology has particular relevance since orchids produce non-endospermic and tiny seeds (Teixeira da Silva 2012). Currently, this particular technology is considered as an effective and alternative method of propagation with higher efficiency in orchids having a higher commercial value, and also as a major tool that can be utilized for the mass propagation of superior and also rarer orchid species with an economic and/or medicinal value. It is precisely described and cited in many literature (Singh et al. 2006; Gantait et al. 2012; Gantait and Sinniah 2013) that synthetic seed technology is an optimistic approach to preserve and clonally propagate superior genotypes, endangered plants with a higher economic value, transgenic plants, and sterile genotypes that can't produce a viable set of seeds, or in the context of orchids, symbiotic association with the beneficial microorganisms is required by means of mycorrhiza in a large scale.

2 Techniques Adopted for Synthetic Seed Development in Orchids

Based on the technology established for orchid so far, propagules under in vitro or in vivo conditions were isolated carefully, and an appropriate hydrogel mainly sodium alginate (0.5–5.0%, w/v) was used. Alginate is structurally aliphatic, hydrophilic, and colloidal in nature (Cameron 2008). Alginate solution is prepared with the help of double-distilled water or by using liquid nutrient medium, and in a form of a bead, it is dropped along with the propagule in calcium chloride ($CaCl_2 \cdot 2H_2O$) or calcium nitrate [$Ca(NO_3)_2$] solution (30–150 mM). Prior to sterilization, the pH of both the complexing agent and alginate matrix is fixed at 5.8. Complexing agent and the gel matrix are sterilized by autoclaving it at 1.06 kg cm^{-2} and 121 °C for 20 min (Gantait and Sinniah 2013). The primary principle regarding encapsulation method using alginate is ion displacement phenomena that take place between sodium (Na^+) and calcium (Ca^{2+}) ions. The rigidity and permeability of the encapsulated propagules depend on concentrations of alginate and calcium chloride which vary in different propagules. According to Teixeira da Silva (2012), to attain the perfect combination for the ideal single-layered synthetic seed formation in hybrid *Cymbidium*, the conditions need to be fulfilled with 3.5% sodium alginate and 100 mM calcium chloride, and the time interval was 40 min. Saiprasad and Polisetty (2003) stated that the amiable conditions for hardening of the encapsulated beads were 3% sodium alginate that formed complex compound with 75 mM calcium chloride for 20–30 min in orchid species like *Oncidium*, *Cattleya*, and *Dendrobium*. Sodium alginate at lower concentrations (1–2%) poses difficulty in encapsulation since it negatively affects the gelling ability, and sodium alginate at higher level of concentrations (5–6%) formed harder, rigid, and isodiametric beads, causing delay in emergence of shoot and roots (Gantait et al. 2012; Gantait and Sinniah 2013). In case of orchids, extensive literature is available on singled-layered synthetic seed. For the development of single-layered synthetic seeds, peristaltic pumps and pipettes are mostly employed (Blandino et al. 2000; Gantait et al. 2012). Constant stirring of solution, where the beads are formed, is done so that cohesiveness among the beads is avoided and spherically shaped beads are formed distinctively. The bead measurements can be altered by changing the innermost diameter of the nozzle of the pipette utilized (Ara et al. 2000). Sterile double-distilled water was used to wash the firm beads, and extra chemicals were removed and placed into a nutrient medium (Gantait et al. 2012; Gantait and Sinniah 2013).

According to Vij and Kaur (1994), synthetic seeds are severely prone to microbial attacks (bacterial or fungal). In order to control the microbial contamination, different antimicrobial agents can be employed. Addition of activated charcoal (AC) that helps in the breakdown of alginate enhances the respiration rate of the propagules, thus significantly extending the storage period (Saiprasad 2001). AC also absorbs the non-desirable and toxic exudates like 5-hydroxymethylfurfural (a toxic byproduct of sucrose formed at the time of autoclaving) (Wang et al. 2007). Hindrances at the time of root and shoot emergence occurs when propagated from synthetic seed that can be managed by self-breaking alginate bead technology (Onishi et al. 1994). Another

demerit of hydrated synthetic seeds is stickiness when exposed in the open air, and it can be solved by coating with Elvax 4260 (ethylene vinyl acetate acrylic acid terpolymer; Dupont, USA) (Redenbaugh and Walker 1990). Till date, there is no report on development of double-layered synthetic seeds or hollow beads in orchid.

3 Orchid Propagules Used for Synthetic Seed Production

In synthetic seed production, different types of propagules are utilized for encapsulation which are further grouped under two major categories.

3.1 Somatic Embryo or Protocorm-Like Body

A somatic embryo possesses both shoot and root propagules, so it can be regarded as a bipolar structure (Standardi and Piccioni 1998). Hence it can be regarded as acceptable propagule for synthetic seed production as compared to conventional propagules, since, in a single step the radicle and the plumule develops simultaneously. In case of orchids, somatic embryo and protocorm-like bodies (PLBs) are identical terms (Texeira Da Silva et al. 2015), where somatic embryos are miniature globule-like structure that ultimately becomes enlarged and later is called "protocorm." Therefore, on the basis of structure, it is termed as "PLB." The morphology of PLB differs from typical somatic embryos by the absence of a distinct embryonic alignment (Norstog 1979). Instead, these are developed with multiple meristematic centers that later transformed into standard embryos, shoots, and roots (da Silva et al. 2000). PLBs consist of shoot apical meristem and leaf primordial and constricted basal tissue, and at primary stages, it exhibited a globular shape, but in advanced stages, a dome shape was exhibited (Kundu and Gantait 2018). The term "PLB" is usually restricted to in vitro culture of orchids (Ishii et al. 1998), apart from a few exceptions (Ilan et al. 1995; Gantait et al. 2012). In general, orchid breeders sprout thousands of seeds in small vessels, under aseptic environment. The ensuing PLBs have an affinity to be transformed into groups of seedlings that are essential to be separated physically. The advances attained by the invention of synthetic seed technology where particular seeds or PLBs were encapsulated in a suitable matrix would obviously minimize the complexity of arrangement and planting of seedlings. Similarly, for the purpose of encapsulation, PLBs could be employed (Fig. 1a). Corrie and Tandon (1993) utilized PLBs from *Cymbidium giganteum* for synthetic seed production. So far, several studies involving PLBs as explants, to develop synthetic seeds of several orchid species were carried out, for instance, *Aranda* × *Vanda*, *C. giganteum*, *Dendrobium wardianum*, *Dendrobium densiflorum*, *Dendrobium nobile*, *Phaius tankervilleae*, *Oncidium*, *Cattleya*, *Spathoglottis plicata*, and *Grammatophyllum scriptum* (Ara et al. 2000; Saiprasad and Polisetty 2003; Vij et al. 2001; Gantait et al. 2012; Mohanty et al. 2013a,b; Pitoyo et al. 2017)

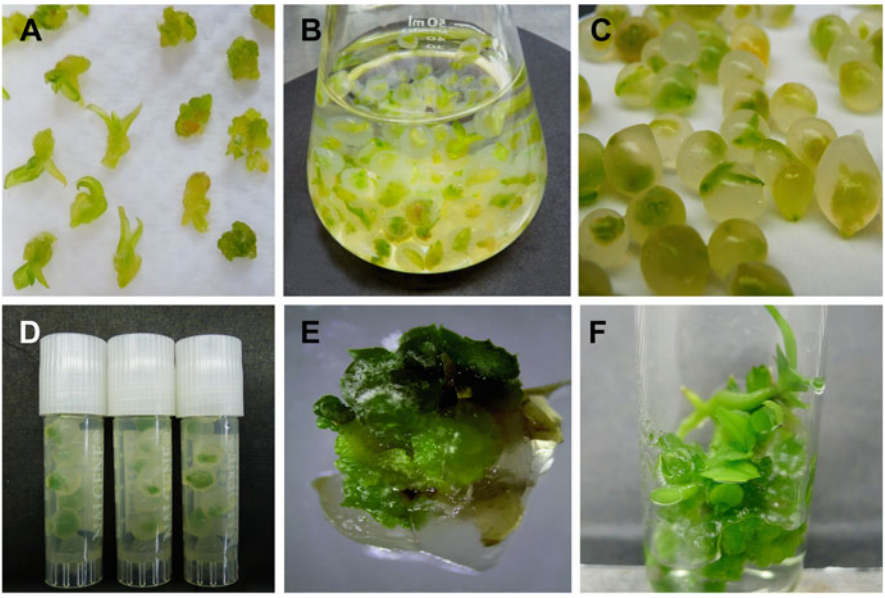

Fig. 1 Synthetic seed production and its application in orchid. (**a**) Shoot tip (left side) and protocorm-like bodies (PLBs) (right side) of *Aranda* Wan Chark Kuan 'Blue' × *Vanda coerulea* Grifft. ex. Lindl., used as explants for encapsulation, (**b**) 3% (w/v) sodium alginate-mounted explants after being dropped in 100 mM calcium chloride solution for formation of synthetic seeds, (**c**) spherical and translucent synthetic seeds, (**d**) storage of synthetic seeds in screw-capped polypropylene tubes in ambient temperature, (**e**) post-storage regeneration (in vitro) of synthetic seeds, (**f**) complete plantlets regenerated from synthetic seeds (figures are not in scale) (Source: Unpublished photographs of Saikat Gantait)

(Table 1). Khor et al. (1998) encapsulated the PLBs of *Spathoglottis plicata* in three ways: firstly, by direct encapsulation without any treatment, secondly, by desiccation prior to encapsulation, and lastly, by the combination of desiccation and abscisic acid (ABA). Henceforth, encapsulation after desiccation increased the viability and survival rate of the protocorms. Datta et al. (1999) encapsulated PLBs from *Geodorum densiflorum* (Lam) Schltr after 30 days of germination from seed. Saiprasad and Polisetty (2003) encapsulated PLBs in *Dendrobium*, *Oncidium*, and *Cattleya* by utilizing shoot tips as explants. Mohanraj et al. (2009) produced synthetic seed of *Coelogyne breviscapa* Lindl by encapsulating PLBs after 60 days of germination from the seed. Sarmah et al. (2010) utilized PLBs from *Vanda coerulea* Griff. ex. Lindl. Leaf explants were used for the culturing of PLBs. Nagananda et al. (2011) conducted an experiment in *Flickingeria nodosa* (Dalz.) Seidenf by culturing PLBs from 45 days of culture. An efficient protocol was developed to induce PLB formation from leaf in orchid hybrid *Aranda* Wan Chark Kuan 'Blue' × *Vanda coerulea* Grifft. ex. Lindl. (Gantait and Sinniah 2012). Using the direct regeneration efficiency of PLBs, the high-rated conversion of synthetic seeds was achieved.

Table 1 Optimal conditions for synthetic seed production and their modes of utilization (propagation or short-term storage or cryopreservation) in different orchid species (arranged in chronological order and alphabetical order within each year)

Species	Explant encapsulated	Sodium alginate (%)	Calcium chloride (mM)	Short-term storage temp. (°C)	Storage duration (days)	Cryopreservation mode	Regeneration/ regrowth (%)	References
Cymbidium giganteum Wall.	PLB	4	100	Not attempted	Not attempted	Not attempted	100 (in vitro), 88 (sand), 64 (soil)	Corrie and Tandon (1993)
Spathoglottis plicata	PLB	2.5	100	Not attempted	Not attempted	Not attempted	100 (in vitro)	Khor et al. (1998)
Geodorum densiflorum (Lam) Schltr.	PLB	4	50	4	120	Not attempted	86 (in vitro), 28 (soil)	Datta et al. (1999)
Ipsea malabarica (Reichb. f.) J. D. Hook.	Bulb	3	7%	Not attempted	Not attempted	Not attempted	100 (in vitro)	Martin (2003)
Dendrobium, Oncidium, and *Cattleya*	PLB	3	75	4	60, 45, and 15, respectively	Not attempted	100 (in vitro)	Saiprasad and Polisetty (2003)
Dendrobium fimbriatum var *Oculatum*, *D. wardianum*, *Cymbidium giganteum*	PLB	2–5	50–150	4	90	Not attempted	30–44 (in vitro)	Kumaria et al. (2005)
Pterostylis saxicola D.L. Jones & M.A.Clem., *Diuris arenaria* D.L. Jones	Seed (with mycorrhizal fungi)	2	100	−18	180	Encapsulation-dehydration	100 (in vitro)	Sommerville et al. (2008)
Coelogynus breviscapa Lindl.	PLB	3	50	4	60	Not attempted	88 (in vitro)	Mohanraj et al. (2009)
Dendrobium candidum Wall. ex Lindl.	PLB	2	50	Not attempted	Not attempted	Encapsulation-vitrification	85 (in vitro)	Yin and Hong (2009)
Vanda coerulea Grifft. ex. Lindl.	PLB	3	100	4	40	Not attempted	41 (in vitro)	Sarmah et al. (2010)

Dendrobium Bobby Messina	PLB	3	100	Not attempted	Not attempted	Encapsulation-dehydration	Unspecified	Antony et al. (2011)
Cymbidium devonianum	PLB	4	100	8	180	Not attempted	100 (in vitro)	Chettri Das et al. (2011)
Phalaenopsis bellina (Rchb.f.) Christenson	PLB	4	75	5	30	Not attempted	65 (in vitro)	Khoddamzadeh et al. (2011a)
Phalaenopsis bellina (Rchb.f.)	PLB	4	75	Not attempted	Not attempted	Encapsulation-dehydration	30 (in vitro)	Khoddamzadeh et al. (2011b)
Flickingeria nodosa (Dalz.) Seidenf.	PLB	2	100	4	60	Not attempted	49 (in vitro)	Nagananda et al. (2011)
Brassidium Shooting Star	PLB	3.5	100	Not attempted	Not attempted	Encapsulation-dehydration	Unspecified	Yin et al. (2011)
Aranda Wan Chark Kuan 'Blue' × *Vanda coerulea Grifft*. ex. Lindl.	PLB	3	75	25	180	Not attempted	76.9 (in vitro)	Gantait et al. (2012)
Dendrobium nobile Lindl.	PLB	3	100	Not attempted	Not attempted	Encapsulation–vitrification, encapsulation-dehydration	75.9 and 50.02, respectively (in vitro)	Mohanty et al. (2012)
Cymbidium pendulum (Roxb.) Sw.	PLB	3	100	4	420	Not attempted	56 (in vitro)	Pehwal et al. (2012)
Cymbidium Twilight Moon 'Day Light'	PLB	3.5	100	Ineffective	Ineffective	Ineffective	100 (in vitro)	Teixeira da Silva (2012)
Dendrobium Shavin White	PLB	3	75	25	135	Not attempted	52 (in vitro)	Bustam et al. (2013)
Cymbidium eburneum Lindl. and *C. hookerianum* Rchb. f.	PLB	3	100	Not attempted	Not attempted	Encapsulation-dehydration	72 and 70, respectively (in vitro)	Gogoi et al. (2013)
Dendrobium nobile Lindl.	PLB	3	100	25	60	Not attempted	78.2 (in vitro)	Mohanty et al. (2013a)

(continued)

Table 1 (continued)

Species	Explant encapsulated	Sodium alginate (%)	Calcium chloride (mM)	Short-term storage temp. (°C)	Storage duration (days)	Cryopreservation mode	Regeneration/ regrowth (%)	References
Dendrobium chrysanthum Wall. ex Lindl.	PLB	3	100	Not attempted	Not attempted	Encapsulation–vitrification	59.9 (in vitro)	Mohanty et al. (2013b)
Aranda Wan Chark Kuan 'Blue' × *Vanda coerulea Grifft*. ex. Lindl.	Shoot tip	3	75	25	200	Not attempted	71.6 (in vitro)	Gantait and Sinniah (2013)
Cymbidium Twilight Moon 'Day Light'	PLB	3.5	100	Not attempted	Not attempted	Encapsulation-dehydration	16 (in vitro)	Teixeira da Silva (2013)
Cymbidium aloifolium (L.) Sw.	PLB	4	200	4	28	Not attempted	97.5 (in vitro)	Pradhan et al. (2014)
Cymbidium aloifolium (L.) Sw.	PLB	4	200	4	90	Not attempted	83.3 (in vitro)	Pradhan et al. (2016)
Spathoglottis plicata Blume.	PLB	3	3% $Ca(NO_3)_2$	4	90	Not attempted	66.7 (in vitro)	Haque and Ghosh (2017)
Grammatophyllum scriptum	PLB	3	75–100	Not attempted	Not attempted	Not attempted	100 (in vitro)	Pitoyo et al. (2017)
Ansellia africana	PLB	3	100	8	75	Not attempted	86.2 (in vitro)	Bhattacharyya et al. (2018)
Cymbidium finlaysonianum Lindl.	PLB	3	100	8	105	Not attempted	44 (in vitro)	Klaocheed et al. (2018)

3.2 Nodes with Apical or Axillary Buds and Microshoots

According to Piccioni (1997), for successful development of artificial seeds, shoot tips and nodal segments are generally used because they ensure higher degrees of genetic stability and somaclonal variations are avoided. The extensive use of plant propagules of non-embryogenic origin (Fig. 1a) is now utilized to produce synthetic seeds in a wide variety of orchid species that has been cited in quite a few literature (Table 1). Lurswijidjarus and Thammasiri (2004) encapsulated shoot tips of *Dendrobium* Walter Oumae. Gantait and Sinniah (2013) used shoot tip explants of *Aranda* × *Vanda* for synthetic seed production from in vitro cultures after 25 days. Although nodal segments are regarded as the most appropriate among the different types of unipolar propagules for encapsulation, there are some shortcomings. Mainly, due to absence of root apex, explants are unable to produce roots. This might be the key reason that limited number of attempts have been made for encapsulation of unipolar plant organs in orchids.

3.3 Meristemoids, Cell Aggregates, and Bulbs

These propagules are complex and heterogeneous. Further, these propagules can differentiate and can develop into different structures like shoots or buds or corm/bulb (Sharma et al. 1992; Tandon et al. 1994; Martin 2003). From different reports it can be highlighted that encapsulation of such propagules should be done prior to their differentiation phase; further it was reported that encapsulation of the proliferating callus showed promising results due to easy regenerative ability and greater potential for transformation. Martin (2003) reported that in vitro-formed bulbs of *Ipsea malabarica* which were encapsulated by sodium alginate were placed in MS medium either on plant growth regulator (PGR)-free ½MS or 6.97 μM kinetin-fortified medium-assisted 100% conversion. Haque and Ghosh (2017) utilized stem discs, leaf tips, and root segments excised from in vitro-grown plants for callus induction in *Spathoglottis plicata* Blume and further encapsulated the PLBs formed from the cultured plants which gave 76.3% conversion (Table 1).

4 Encapsulating Agent and Matrix Used for Synthetic Seed Production in Orchid

Synthetic seed matrix is the key component that surrounds the explants and thus demonstrates its significant influence on the ultimate sustainability of synthetic seed (Gantait et al. 2015; Pitoyo et al. 2017). It is indispensable that the artificial seed coat shields the explants during its storage and handling and simultaneously retains the effectiveness to hold artificially provided growth factors (Khor and Loh 2005). The

soft hydrogel protects the explant by making sure that the least amount of pressure be put on the same, thus assuring a much lesser damage to the plant material. In the perspective of hydrogel, the explants are commingled with a polymeric solution that when dropped in the other liquid comprising divalent metal ions, commenced a cross-linking effect, giving rise to the hydrogel. The resultant encapsulation beads held the explants firmly and continued to provide sufficient resistance to exterior mechanical stress, for easy handling (Gantait et al. 2015).

It is obvious that the choice of suitable explants is the core of the notion of synthetic seed, but the selection of related matrix elements to be used in blend with the biological substances is similarly crucial. The concentration and category of gel essential for encapsulation and the degree of exposure of encapsulated seeds to $CaCl_2 \cdot H_2O$ are vital as well (Redenbaugh et al. 1991, 1993). Coating agents are of various types like sodium pectate, sodium alginate, potassium alginate, sodium alginate with carboxymethyl cellulose, carrageenan, agar, gelrite, gelatin, tragacanth gum, guar gum, etc. which have also been used as hydrogels as mentioned by Ara et al. (2000) and Rai et al. (2009). According to Redenbaugh et al. (1987), sodium alginate and calcium salt serve as the best combination for encapsulation due to non-damaging property of these ions, lower price, easy handling, and high plantlet conversion rate. The gel circumventing the capsule has a potential to provide a range of nutrients that helps in enhancing the survivability and increasing the rate of growth in embryos. Sodium alginate is now selected and utilized frequently in all experiments because of moderate level of viscosity, low spinning potentiality of solution, negligible toxicity of propagules, instant gellation, cheaper price, and its biocompatibility (Saiprasad 2001).

A number of gel types are utilized for encapsulation; nevertheless, sodium alginate is recognized to be the most commonly used matrix because of its minimum cost, gelling nature, and nonhazardous property (Cheruvathur et al. 2013). Alginate is the favored choice since it enhances capsule formation, and, additionally, the sturdiness of alginate beads reassures a much-improved safeguard (in comparison with agar) to protect explants from physical injury (Saiprasad and Polisetty 2003).

With the times, the idea of matrix materials has advanced into a reasonably refined interaction that centers on the transformability of synthetic seeds. It is apparent from the current chapter that a greater part of the optimal results in terms of well-developed round beads was formed with 100 mM $CaCl_2 \cdot H_2O$ and 3% sodium alginate (Table 1), for example, in *Vanda coerulea* (Sarmah et al. 2010), *Cymbidium devonianum* (Chettri Das et al. 2011), *Cymbidium pendulum* (Roxb.) Sw. (Pehwal et al. 2012), *Dendrobium nobile* (Mohanty et al. 2013a), *Dendrobium chrysanthum* Wall. ex Lindl. (Mohanty et al. 2013b), *Cymbidium eburneum* Lindl. and *C. hookerianum* Rchb. f. (Gogoi et al. 2013), *Aranda* Wan Chark Kuan 'Blue' × *Vanda coerulea Griff*. ex. Lindl. (Gantait et al. 2012; Gantait and Sinniah 2013), *Cymbidium finlaysonianum* Lindl. (Klaocheed et al. 2018) etc., and the regeneration of synthetic seeds was ~90% in most of the occasions. Apparently, 3% sodium alginate solution and 100 mM $CaCl_2$ H_2O assisted in the most beneficial ion exchange including Na^+ and Ca^{2+}, forming compact, translucent, isodiametric beads. Reduced levels (1 and 2%) of sodium alginate were not suitable since the

capsules were of irregular form and remarkably brittle and soft to grip; at the same time, at raised levels (4 and 5% sodium alginate), the capsules were too inflexible (Fig. 1) causing substantial obstruction during conversion of synthetic seeds.

5 Modes of Utilization of Orchid Synthetic Seeds

In the field of orchid biotechnology, synthetic technology has opened up new dimensions. For the management of plant germplasm, the utilization of synthetic seeds can be done in several ways.

5.1 In Vitro Plant Production

According to Mandal et al. (2000) as well as Pinker and Abdel-Rahman (2005), under in vitro conditions, the encapsulation technique functions as the most conventional and genuine means for producing plant propagules (Table 1). At the same time, the semisolid culture medium or planting substrates (that includes perlite, vermiculite, vermin compost, soilrite, soil, sand, and gravel) aid as efficient means for the successful conversion of synthetic seeds. Generally, for accomplishing higher conversion efficiency, nutrient-rich media are frequently preferred over nutrient-deficit media (Mandal et al. 2000). Lurswijidjarus and Thammasiri (2004) cultured encapsulated shoot tips of *Dendrobium* Walter Oumae in Vacin and Went (VW) media fortified with agar and sucrose. In the nutrient medium, the PGR dosage is variable in different plant species. For example, when shoot tips and PLBs were encapsulated and placed into MS medium which was supplemented with PGRs, a high (>90%) conversion efficiency was achieved. Nagananda et al. (2011) cultured PLBs in Burgeff's N3F basal medium consisting 2% sucrose and agar. The same was fortified with adenine sulphate and IAA. Pradhan et al. (2014) recounted that full strength of MS medium provided the highest percentage of recovery of plantlets in *Cymbidium aloifolium.* It was also supplemented with 0.5 mg/L NAA and BAP. Bhattacharyya et al. (2018) conducted an experiment in *Ansellia africana* (Leopard orchid) where culture was done in rooting media comprising of indole acetic acid (IAA) and indole butyric acid (IBA) (5–20 μM). Phenolic elicitors phloroglucinol (PG) (10–40 μM) was added in order to reduce the effects of hyper-hydricity and to eradicate the hindrances that surfaced at the time of root induction and branching. The highest conversion frequency was achieved at the concentrations of 15–20 μM IAA and IBA along with 40 μM PG. During in vitro synthetic seed conversion, the gelling agent also serves as an essential factor. The most popular gelling agent utilized for synthetic seed conversion medium is agar (Cameron 2008). Alternatively, Singh (2008) demonstrated that conversion results of synthetic seed by the application of phytagel gave more promising results than agar.

5.2 Direct Sowing

Sowing of synthetic seeds under ex vitro conditions provides an economical technique for direct production of the plantlets in terms of economical aspect thus positively enhancing the large-scale production of plant species. The acclimatization procedure can also be skipped when direct or ex vitro sowing is done. Mandal et al. (2000) suggested that for successful commercial-scale propagation, simple and economical substrate such as soilrite, soil, sand, or vermi-compost is essential for achieving maximum conversion percentage in the plantlets. The feasibility of direct germination of artificial seeds of *Dendrobium* Shavin White was tested in different substrates, namely, semisolid ½ Murashige and Skoog (1962) basal medium, sterilized liquid ½ MS basal medium-drenched cotton bed, sterilized distilled water-drenched cotton bed, and cotton-drenched with non-sterile water-drenched cotton bed (Bustam et al. 2013). Chettri Das et al. (2011) reported that acclimatization of *Cymbidium devonianum* with higher survivability percentage can be attained by using a potting mixture comprising of brick, charcoal, decayed waste, and peat moss. The addition of litter and moss heightened the survivability rate due to better drainage and air circulation.

The major bottleneck that reduces the conversion efficacy is nutrient deficiency, so in order to tackle this problem, endogenous or exogenous application of nutrients is required. In addition, at the time of direct sowing, microbial incidence and attack serve as the biggest obstacles; thus commercialization of encapsulation technology becomes difficult. Severe contamination is caused by the release of organic nutrients, mainly sucrose-stimulated microbial incidence that inhibits rooting (Nhut et al. 2005). However, Nhut et al. (2005) developed a conventional method in *Cymbidium* wherein, a fungicidal solution, namely, chitosan, was used to coat the synthetic seeds.

5.3 Short-Term Germplasm Conservation

Artificial seed technology positively implements the germplasm exchange. For this reason, the storage conditions play a significant role in determining the successful conversion rate of synthetic seed at the time of transporting it to long distance (Fig. 1d). Therefore, definite storage period and appropriate storage conditions serve as the primary criteria to maintain viability of synthetic seed mainly during transportation. In most of the literature, the perfect temperature for storing synthetic seed is 4–5 °C (Saiprasad and Polisetty 2003; Teixeira da Silva 2012) (Table 1). Khoddamzadeh et al. (2011a) encapsulated and stored PLBs of *Phalaenopsis bellina* under storage temperatures of 5, 15, and 25 °C where higher viability and survivability were achieved at temperature of 5 °C since, there is an inverse relation between storage temperature and viability. With the increase in storage temperature, there is reduction in viability. Chettri Das et al. (2011) conducted an experiment in *Cymbidium devonianum*, where PLBs were encapsulated and stored in temperature

(0, 4, and 8 °C) under dark conditions. High survival rate was observed in temperatures 4 and 8 °C. The storage duration can also be extended up to 180 days with minimum loss in viability. Similarly, Klaocheed et al. (2018) encapsulated and stored PLBS of *Cymbidium finlaysonianum* Lindl at temperature ranges of 0 ± 2, 4 ± 2, 8 ± 2, and 25 ± 2 °C at storage periods of 15, 30, 45, 60, 75, 90, 105, and 120 days. High conversion frequency was achieved in 8 ± 2 °C as compared to other temperature conditions. Subsequently, Bhattacharyya et al. (2018) reported similar results in *Ansellia africana* (Leopard orchid) where synthetic seeds were maintained under temperatures of 4, 8, and 25 °C for 15, 30, 45, 60, 75, and 90 days. The best results were obtained at 8 °C and at 75 days. A conversion frequency of 86.21% was achieved. In contrast to these reports, there are multiple instances wherein higher temperature (preferable ambient room temperature) proved to be efficient in sustaining the storability of orchid synthetic seeds. In an experiment conducted by Bustam et al. (2013) in *Dendrobium* Shavin White, the encapsulated PLBs were stored under a range of temperatures (4, 10, 25, and 30 °C) for 135 days in different tiers of a freezer as well as in the growth room. The results showed that at 25 °C temperature, the highest conversion frequency was exhibited by the beads. In correspondence with this report, Gantait et al. (2012) and, later, Gantait and Sinniah (2013) optimized the storage condition of *Aranda* × *Vanda* synthetic seeds (derived from PLBs and shoot tips) at 25 °C with germination (Fig. 1e) efficiency of as high as ~72–77% after 180–200 days of storage.

5.4 Cryopreservation: Long-Term Conservation of Germplasm

Cryopreservation is an apt method for long-term germplasm conservation of plant propagules under ultralow temperature conditions for a larger span (Gantait et al. 2017). It ensures the integrity and stability of the germplasm on the basis of its genotype and phenotype (Texeira Da Silva et al. 2014). Due to orchid's miniature structure and thin seed coat, it imparts formidable opportunities for cryopreservation (Merritt et al. 2014). PLBs of *Dendrobium candidum* (Yin and Hong 2009) were efficiently cryopreserved with the aid of encapsulation-vitrification technique. Khoddamzadeh et al. (2011b) developed a convenient technique of encapsulation-dehydration in *Phalaenopsis bellina* where the PLBs were used and no expensive programmable freezer and higher levels of injurious cryoprotectants were required. This strategy is based on consecutive dehydration of plant cells in sucrose-rich medium osmotically due to the gradual loss of water from encapsulated propagules. Similarly, Yin et al. (2011) used encapsulation dehydration technique to cryopreserve the PLBs of *Brassidium* Shooting Star orchid hybrid, where the PLBs were successfully dehydrated using preheated silica gel in laminar air flow cabinet and finally stored in liquid nitrogen for 24 h. Subsequent culturing and biochemical assays displayed some significant results, which revealed that cryopreserved PLBs

produced lesser chlorophyll and minimum protein content, however, high peroxidase activity was also observed. It was also conducted by Antony et al. (2011) in *Dendrobium* Bobby Messina where the PLBs were also cryopreserved using encapsulation-dehydration technique using oven-satirized silica gel in paraffin-sealed wax glass. Mohanty et al. (2011) established protocols for both encapsulation-dehydration and encapsulation-vitrification. In case of dehydration, it was done in laminar air flow, and on the other hand, the vitrification was done using a vitrification solution (VS) that comprised of 30% glycerol, 15% ethylene glycol, and 15% dimethyl sulfoxide (DMSO) and 0.4 M sucrose. After treatment with VS, it was immersed in liquid nitrogen. The results showed that encapsulation-vitrification showed better conversion efficiency (75.92%) and high survival rates as compared to encapsulation dehydration (53.3%). Later on, the same group of authors (Mohanty et al. 2013b) established a protocol for cryoconservation of a rare orchid *D. chrysanthum* via synthetic seed production of PLBs and their simultaneous long-term conservation by encapsulation-vitrification approach. Following an initial osmoprotection (with a mixture of 2 M glycerol and 0.4 M sucrose in the encapsulation matrix followed by preculture and loading treatment for 80 min), the synthetic seeds (encapsulated PLBs) were subjected to PVS2 (Sakai and Kobayashi 1990) for 100 min. Exposure of encapsulated-vitrified PLBs to liquid nitrogen and subsequent revival showed as high as 63.2% survival and 59.9% regrowth. Gogoi et al. (2013) modified the encapsulation-dehydration protocol in *Cymbidium eburneum* Lindl., and *Cymbidium hookerianum* Rchb. f. where the encapsulated beads were treated using sucrose (at varying concentrations 0.3–0.8 M) for 20 h prior to dehydration in laminar air flow cabinet, and then cryopreserved. The results revealed that 0.7 M sucrose showed the highest conversion efficiency. Pretreatment with sucrose prevents freezing injuries involved during the desiccation. Teixeira da Silva (2013) utilized the encapsulation-vitrification method in hybrid *Cymbidium*, where encapsulated PLBs were treated with vitrification solutions. DMSO, mannose, and PEG-6000 resulted in a negative impact on the explant survivability, but 2% sucrose accounted for the best results. However, hyper-hydricity effects were not reported from any of the abovementioned osmoprotectants. For majority of orchid species, encapsulation-dehydration has been extensively used for cryopreservation of the propagules, mainly shoot tips and PLBs. A remarkable conclusion is drawn out to the fact that these protocols resulted in more than 80% survivability in the propagules. Encapsulation-vitrification and encapsulation-dehydration are regarded as simple and cheaper techniques that maintain the genetic stability and minimize the chances of potential injuries (Moges et al. 2004).

6 Molecular Marker Technology in Orchid Synthetic Seed

According to Hirai and Sakai (2000), Scocchi et al. (2004), and Bekheet et al. (2007), the evaluation of genetic variability in the propagules generated from conserved synthetic seeds has drawn much interest. The evaluation of genetic

stability is mandatory for the proper utilization of synthetic seed in germplasm conservation and exchange (Dehmer 2005). According to Jokipii et al. (2004), Borner (2006), Mandal et al. (2007), and Agnihotri et al. (2009), the molecular marker technology is now an upcoming advanced practice to sample the germplasm systematically and further analyze their molecular status. Random amplified polymorphic DNA (RAPD) and inter simple sequence repeats (ISSR) analyses have widely been used for studying clonal integrity, uniformity, and detection of somaclonal variations on genetic basis due to easy handling, wider availability, lower cost, and simple genotyping. Poobathy et al. (2013) assessed genetic stability of encapsulated-dehydrated *Dendrobium* Sonia-28 PLBs using RAPD marker and reported that the regenerants were genetically polymorphic (with similarity index of 0.4–1), when compared to the stock culture. Gantait and Sinniah (2013) employed ISSR markers in *Aranda* × *Vanda* to deduce the genetic fidelity and uniformity of synthetic seeds of the plantlets (shoot tips were encapsulated) derived from the mother plant. For this experiment nine ISSR markers were used; it was observed that a total of 561 bands, which were all scorable, were produced with ~5.5 bands per primer. None of the bands were polymorphic (out of the 561 scorable bands). It was concluded that the 100% similarity coefficient was revealed thus confirming the molecular stability of *Aranda* × *Vanda* plants, regenerated from synthetic seeds. Lately, Worrachottiyanon and Bunnag (2018) reported clonal fidelity of *Cymbidium finlaysonianum* plantlets, regenerated after encapsulation-dehydration-based cryopreservation. RAPD analysis (with 45 primers) employed for comparison of the polymorphic bands between plantlets germinated from non-cryopreserved and cryopreserved synthetic seeds revealed a similar index value of 0.998 (considered to be close to 1), indicating the genetic stability of the cryopreserved synthetic seeds (encapsulated protocorms). Most recently, Bhattacharyya et al. (2018) assessed the clonal fidelity of *Ansellia africana* (Leopard orchid) plantlets, developed from encapsulated micro-shoots along with the designated mother plant using start codon targeted (SCoT) and inter-retrotransposon amplified polymorphism (IRAP) marker systems. Both markers exhibited a great extent of genetic homogeneity within the in vitro-raised plants, wherein 5 IRAP and 8 SCoT primers developed 81 bands, respectively, of which only 6 bands were polymorphic. So far there are fewer reports on molecular marker-assisted genetic fidelity assessment of synthetic seed-germinated orchid plantlets, and hence, there is still much to be done in this field.

7 Problems, Limitations, and Future Prospects

In the recent era, synthetic seed technology is getting immensely popular for orchids due to its extensive applications, considering the facets of germplasm storage, conservation, and exchange between different countries and research institutes. Apart from the achievements, there are some shortcomings required to be solved in order to commercialize this technology for orchid. Firstly, in order to commercialize

synthetic seed technology, cheaper methods should be employed to enhance the production of highly viable and better-quality micropropagules. Secondly, several problems that are associated with the usage of somatic embryos in the production of synthetic seed such as reduction of embryogenic vigor in old cultures, indeterminate development, advanced germination, structural incongruities, failure to withstand desiccation tolerance, etc. should be resolved (Ara et al. 2000). A solution to this problem had been suggested (Soneji et al. 2002) which is the usage of non-embryogenic propagules instead of somatic embryos or PLBs which is favorable in many other plant species that produce nonviable set of seeds, although in some orchid species, usage of unipolar propagules leads to absence of root apex in the explants. Thirdly, there are still many challenges to make the technique more practical and feasible especially for the farmers and producers; mainly the ex vitro techniques or the direct sowing of seeds needs to be refined (Jung et al. 2004). The key shortcomings associated with the ex vitro techniques are reduction in survivability rate arising from nutrient deficiency and oxygen supply and microbial attack that needs major attention (Nhut et al. 2005). Lastly, alginate encapsulation by conventional method is a labor-intensive process which involves repetitive handling of the explants in various stages that includes excision, alginate coating being dipped into calcium chloride solution, rinsed with water and finally place into a vessel. West and Preece (2009) proposed a solution to this problem by signifying the usage of bulk encapsulation approach that reduced the labor and improved the efficacy. Still, bulk alginate encapsulation has many shortcomings; plantlets when exposed to high sodium alginate concentration leads to reduction in shoot and root growth and matrix shrinkage.

The above listed shortcomings are required to be surveyed extensively and solved so that this technology can be utilized in a long-term basis keeping in mind the principles of sustainable development and conservation. To refine the methods, protocols and machinery that are required for handling synthetic seed at the production and post-production stages, additional monetary support and investments are essential. Minimal progress has been made in the field of synthetic seed technology in orchid. Attempts were made to demonstrate the feasibility of synthetic seed production in orchids in various literatures listed in this chapter. Still, there is need to implement this technology in a commercial and broad scale so that the concept can emerge from the theoretical means and can be more conceptualized in practical basis.

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Compliance with Ethical Standards

Conflict of Interest Declared none.

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Somatic Embryos Encapsulation for Synthetic Seed Production of Sugar Palm (*Arenga pinnata* Wurmb Merr.)

Nazatul Asikin Muda and Asmah Awal

Abstract This paper describes a protocol for producing synthetic seeds of sugar palm (*Arenga pinnata* Wurmb Merr.) using encapsulated somatic embryos (SEs) and secondary somatic embryos (SSEs) at different developmental stages. The study investigates in vitro germination response of the synthetic seeds influenced by different concentrations of encapsulation matrix and its viability at storage temperature of 4 °C and 25 °C. Encapsulation of SEs and SSEs in 3.0% sodium alginate, complexed in 100 mM calcium chloride sterile solution ($CaCl_2 \cdot 2H_2O$) and inoculated on basal MS media under dark condition at 25 ± 2 °C, promoted an optimum 30 and 80% germination rate after 4 and 8 weeks, respectively. Germinated synthetic seeds transferred to MS + 1.0 mg/L BAP (6-Benzylaminopurine) + 1.0 g/L NAA (1-Naphtaleneacetic acid) promoted an optimum average number of shoot regeneration at 7.75 ± 1.32 after 12 weeks. Synthetic seeds being cold-stored at 4 °C displayed consistent declination rate of germination at 0–120 days of storage, while the synthetic seeds stored at normal culture condition of 25 °C promoted optimum germination (80%) during 0–45 days of storage. Optimum number of shoot regeneration at 11.00 ± 0.91 and 12.25 ± 1.32 with the average number of roots at 3.00 ± 0.41 was recorded from the non-refrigerated synthetic seeds cultured on MS + 1.0 mg/L BAP + 1.0 mg/L NAA after 8 weeks. Shoots of normal morphology were observed after 12 weeks of transfer on basal MS media.

Keywords $CaCl_2 \cdot 2H_2O$ · Sodium alginate · Somatic embryos · Sugar palm (*Arenga pinnata* Wurmb Merr.) · Synthetic seeds

N. A. Muda
Faculty of Plantation and Agrotechnology, Universiti Teknologi MARA, Shah Alam, Selangor, Malaysia

A. Awal (✉)
Agricutural Biotechnology Research Group, Faculty of Plantation and Agrotechnology, Universiti Teknologi MARA, Shah Alam, Selangor, Malaysia
e-mail: asmah138@uitm.edu.my

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Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
$2H_2O$	Sterile distilled water
BAP	6-Benzylaminopurine
$CaCl_2 \cdot 2H_2O$	Calcium chloride sterile solution
EtOH	Ethyl alcohol
MS	Murashige and Skoog medium
NAA	1-Naphthaleneacetic acid
$NaCl_2$	Sodium hypochlorite
PGRs	Plant growth regulators
SEs	Somatic embryos
SSEs	Secondary somatic embryos

1 Introduction

The idea of synthetic seeds, also known as "synseed," was first officially presented by Toshio Murashige in 1977 at a tissue culture symposium for horticultural purposes in Belgium (Magray et al. 2017). Murashige (1977) described synthetic seeds as "an encapsulated single somatic embryo" which was later defined by Gray et al. (1991) as "an encapsulated single somatic embryo that is engineered for the practical use in commercial plant production."

As the technology started to gain interest from researchers throughout the century, the concept of synthetic seeds was later discovered to be limited only in plants in which somatic embryos could be demonstrated. Due to recalcitrance issues in certain plant species to develop somatic embryogenesis, the synthetic seed concept was later broadened as the "encapsulation of a range of in vitro-derived propagules" (Bapat et al. 1987). However, the true extended definition concept of synthetic seed technology was actually described by Kamada (1985) who indicated the encapsulation of other vegetative parts, tissues, and cell aggregates of plants aside from somatic embryos in an artificial coating for sowing as a seed and that have the ability to convert into a complete plant under in vitro and ex vitro condition and that also retaining this conversion potential after storage. This concept was afterward pioneering a large number of synthetic seeds studies of many plant species belonging to both monocot and dicot groups (Purohit 2013).

Commercially valuable crops which have a strong technological basis such as those that are capable of producing high-quality somatic embryos are a great candidate for the implementation of synthetic seeds (Redenbaugh et al. 1987). Transgenic plants, elite plant species, non-seed-producing plants, and plant lines that have a problem in seed propagation are another significant crop characteristic which demanded the application of this clonal technique. Synthetic seed technology symbolizes the biotechnology advancement in agriculture in a cost-effective way as it shortened the laborious procedure of conventional recombination breeding system

for many plant species (Rihan et al. 2017). Among other great advantages of this technique also include cheaper plantlet cost while maintaining its clonal nature, simple methodology with high potential for mass propagation of elite plant varieties, facilitating direct use of in vitro plantlets to ex vitro conditions, long-term storage potential without losing viability, easy long-distance transportation, and ease of handling while in storage (Bhatia and Bera 2015).

Sugar palm which belongs to monocot plant group is an economically important palm species cultivated primarily in the Southeast Asian regions for its sugary sap and industrially strong black fibers (Mogea 2003). The implementation of synthetic seeds in sugar palm was to overcome the complex handling and recalcitrancy of natural seeds for germination (Asikin and Puspitaningtyas 2000). Long dormancy period and short-storage life are other concerning issues for successful seed propagation of sugar palm (Soeseno 2000; Orwa et al. 2009).

The current study thus reports the synthetic seed establishment through the encapsulation of SEs and SSEs at torpedo-cotyledonary developmental stage obtained from immature zygotic embryo explants of sugar palm. The method of inducing the somatic embryos in vitro as propagules was also described. The established synthetic seed protocol was hoped to facilitate mass propagation of clonal sugar palm seedlings for commercial production while conserving its germplasm for effective and systematic use at any time in the future.

2 Materials and Methods

2.1 Preparation of Plant Materials and Surface Sterilization Method

Fresh fruits of sugar palm at an approximate average size of 5.5 cm were collected from a plantation in Raub, Pahang, Malaysia, and cleaned under running tap water. Surface sterilization method was carried out by soaking the washed fruits with 70% ethyl alcohol (EtOH) for 30 min, rinsed with sterile distilled water ($2H_2O$) three times, and followed with 30-min immersion in 50% sodium hypochlorite ($NaCl_2$) added with a few drops of Tween 20. The surface-sterilized fruits were later rinsed with another three changes of $2H_2O$ prior to drying in a laminar growth chamber. Immature zygotic embryos extracted from the endosperms of the surface-sterilized sugar palm fruits were used as explants to initiate somatic embryogenesis under in vitro condition.

2.2 Preparation of Culture Media

MS (Murashige and Skoog 1962) media were prepared with the mixture of 4.41 mg/L MS salt and 30.0 g/L sucrose and solidified with 2.5 g/L gelrite. The pH of all culture

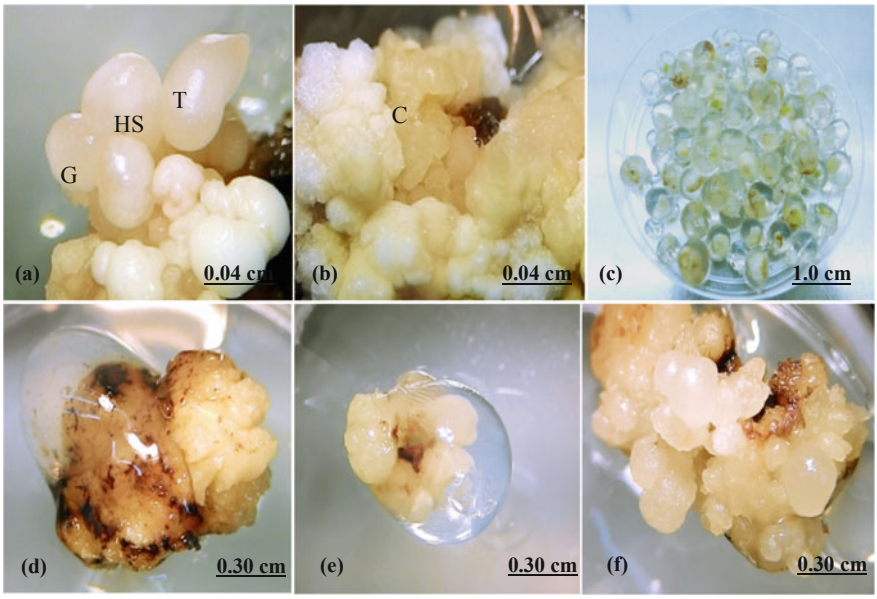

Fig. 1 SEs at (**a**) globular [G], heart-shaped [HS], torpedo [T], and (**b**) cotyledonary [C] developmental stage as propagules for synthetic seeds production of sugar palm. (**c**) Encapsulated SEs and SSEs in optimum encapsulation matrix (3.0% sodium alginate +100 mM $CaCl_2 \cdot 2H_2O$). (**d**) Secondary callus and (**d**) SSE development after 4–8 weeks of culture on basal MS media. (**e**) Microshoot establishment after 2 weeks on MS + 1.0 mg/L BAP + 1.0 mg/L NAA under dark condition at 25 ± 2 °C

media involved in the experiment was adjusted at 5.8 and sterilized at 121 °C for 20 min. Plant growth regulators (PGRs) were added accordingly to the objectives of the experiments.

2.3 Somatic Embryogenesis Induction

Immature zygotic embryo explants were cultured on MS media supplemented with 0.4 mg/L 2,4-D (2,4-dichlorophenoxyacetic acid) + 0.5 mg/L NAA to induce primary embryogenic calluses under in vitro condition at 25 ± 2 °C in a complete darkness. Under similar incubation environment, the induced primary calluses were later transferred on a fresh MS medium containing 60.0 g/L sucrose + 0.4 mg/L 2,4-D + 0.5 mg/L NAA + 3.0 g/L casein hydrolysate to initiate somatic embryogenesis at globular, heart-shaped, and torpedo developmental stage. SSEs at torpedo-cotyledonary developmental stage were demonstrated within 4 weeks prior to transfer of SEs on MS + 0.4 mg/L 2,4-D + 0.5 mg/L BAP + 1.0 mg/L silver nitrate (Muda and Awal 2017). Developed translucent SEs and SSEs at an approximate size of 3.0–4.0 mm (Fig. 1a, Fig. 1b) were used as propagules to develop synthetic seeds of sugar palm.

2.4 Encapsulation of SEs and SSEs for Synthetic Seed Production

The encapsulation procedure of SEs and SSEs for synthetic seed production of sugar palm was carried out as demonstrated by Awal et al. (2008). Established SEs and SSEs were extracted as individuals and encapsulated in a matrix solution consisted of 30.0 g/L sucrose, four different concentrations of sodium alginate (1.0, 2.0, 3.0, and 4.0%), and PGRs (1.0 mg/L BAP + 1.0 mg/L NAA). The matrix solutions were autoclaved at 121 °C for 20 min. Each SEs and SSEs was dropped in the cooled-down sodium alginate solutions and later pipetted into sterile solutions of three different concentrations of $CaCl_2 \cdot 2H_2O$ (50.0 mM, 75.0 mM, and 100.0 mM). The sterile solutions of $CaCl_2 \cdot 2H_2O$ were kept stirred to ensure thorough coating of propagules. After 15 min, formed synthetic beads were collected with a sieve, rinsed with $2H_2O$, and desiccated on sterile distilled paper prior to in vitro culture on basal MS media for germination.

2.5 In Vitro Germination of Synthetic Seeds and Storage

Encapsulated SEs and SSEs as synthetic seeds were cultured on basal MS media prior to incubation in a growth chamber fixed at the temperature of 25 ± 2 °C under dark condition. Their germination percentage and days taken for the germination to take place were weekly observed and recorded at 4-week intervals. The germination performance of the synthetic seeds being cold-stored at 4 °C was also recorded for 4 months (120 days). Synthetic seeds stored at normal culture environment (25 °C) served as control treatment.

2.6 Experimental Design and Data Analysis

The experiments were laid out using randomized complete block design (RCBD) and repeated twice with ten replications for each treatment. The germination rate was calculated as the percentage of synthetic seeds germinated from the total number of synthetic seeds cultured in vitro. The frequency of shoots and roots observed from the experiment was recorded and analyzed using analysis of variance (ANOVA) followed with Tukey post hoc test at a significant level of 0.05 ($P < 0.05$) using SPSS (Statistical Package for the Social Sciences) software version 20.0. Results were expressed as mean ± standard error (SE).

3 Results and Discussion

The method to encapsulate somatic embryos to produce artificial seeds has been demonstrated in various monocotyledonous plant species which included rice (Roy and Tulsiram 2013; Kumar et al. 2005), maize (Thobunluepop et al. 2005), oil palm

(Mariani et al. 2014; Inpuay and Te-Chato 2012), and date palm (Bekheet 2017; Diab and El-Fadl 2016), but none has ever been reported in sugar palm. Thus, in the present work, a protocol to establish synthetic seeds of sugar palm through the encapsulation of somatic embryos (SEs) and secondary somatic embryos (SSEs) had been conducted. The selection of somatic embryos as propagules for the purpose of establishing synthetic seeds is significant to the fact that they are structurally similar to zygotic embryos found in seeds and feature many of their useful characteristics, including the ability to grow into complete plants (Saiprasad 2001).

The establishment of decent synthetic seeds of sugar palm was dependent on the proper manipulation of sodium alginate and $CaCl_2 \cdot 2H_2O$ as encapsulation matrix. Table 1 showed the morphology of synthetic seeds produced using different concentrations of sodium alginate and $CaCl_2 \cdot 2H_2O$ as encapsulation matrix. Soft, solid, uniform, and round-shaped translucent synthetic beads were obtained from the encapsulation of the selected propagules in 3.0% sodium alginate and 75–100 mM $CaCl_2 \cdot 2H_2O$ (Fig. 1c). Meanwhile, synthetic seeds formed with lower concentrations of sodium alginate at 1.0–2.0% and complexed with all tested concentrations of $CaCl_2 \cdot 2H_2O$ (50 mM, 75 mM, and 100 mM) were observed to produce very delicate/delicate, fragile, and translucent beads with indefinite shapes. Given a slightly forced strength would easily rupture and break them. On the other hand, higher concentrations of sodium alginate at 4.0% and 50–100 mM $CaCl_2 \cdot 2H_2O$ produced hard, mostly rounded, and tail-shaped translucent synthetic beads. A similar result was reported by Tarmizi and Zaiton (2013) in their attempt to establish synthetic seeds for oil palm.

Table 1 The morphology of sugar palm's synthetic seeds formed by different concentrations of sodium alginate and $CaCl_2 \cdot 2H_2O$ as encapsulation matrix

Sodium alginate (%)	$CaCl_2 \cdot 2H_2O$ (mM)	Synthetic bead morphology
1.0	50	Very delicate, fragile, translucent, and indefinite-shaped beads
	75	Very delicate, fragile, translucent, and indefinite-shaped beads
	100	Very delicate, fragile, translucent, and indefinite-shaped beads
2.0	50	Delicate, translucent, and indefinite-shaped beads
	75	Delicate, translucent, and indefinite-shaped beads
	100	Delicate, translucent, and indefinite-shaped beads
3.0	50	Soft, solid, uniform, round-shaped, and translucent beads
	75	Soft, solid, uniform, and round-shaped translucent beads
	100	Soft, solid, uniform, and round-shaped translucent beads
4.0	50	Hard, mostly rounded, and tail-shaped translucent beads
	75	Hard, mostly rounded, and tail-shaped translucent beads
	100	Hard, mostly rounded, and tail-shaped translucent beads

In terms of germination potential, it was observed that the synthetic seeds produced with different consistencies of encapsulation matrices promoted a significantly different rate of germination when inoculated on basal MS media under in vitro condition (Table 2, Table 3). Optimum germination rate of 30% at 4 weeks and 80% at 8 weeks of culture was recorded from the synthetic seeds formed with 3.0% sodium alginate and 100 mM $CaCl_2 \cdot 2H_2O$. This result then followed with 30%

Table 2 The germination response of the synthetic seeds formed with different concentrations of sodium alginate and $CaCl_2 \cdot 2H_2O$ inoculated on basal MS media after 4 weeks

		4 weeks	
Sodium alginate (%)	$CaCl_2 \cdot 2H_2O$ (mM)	Germination rate (%)	Regenerant type
1.0	50	10	Callus
	75	20	Callus
	100	–	–
2.0	50	10	Callus
	75	20	Callus
	100	–	–
3.0	50	30	Callus
	75	–	–
	100	30	Callus
4.0	50	10	Callus
	75	–	–
	100	–	–

Table 3 The germination response of the synthetic seeds formed with different concentrations of sodium alginate and $CaCl_2 \cdot 2H_2O$ inoculated on basal MS media after 8 weeks

		8 weeks	
Sodium alginate (%)	$CaCl_2 \cdot 2H_2O$ (mM)	Germination rate (%)	Regenerant type
1.0	50	20	Secondary somatic embryos
	75	20	Secondary somatic embryos
	100	20	Secondary somatic embryos
2.0	50	50	Callus, secondary somatic embryos
	75	10	Callus
	100	60	Secondary somatic embryos
3.0	50	60	Callus
	75	20	Callus
	100	80	Callus, secondary somatic embryos
4.0	50	20	Callus, secondary somatic embryos
	75	30	Callus
	100	50	Callus

and 60% germination rate after 4 and 8 weeks, respectively, from the synthetic seeds formed with 3.0% sodium alginate and 50 mM $CaCl_2 \cdot 2H_2O$. The lowest germination rate (10%) was recorded from the synthetic seeds formed with 4.0% and 100 mM $CaCl_2 \cdot 2H_2O$ after 8 weeks of culture. Not germinated synthetic seeds were observed to turn severely browny, watery, and mostly deteriorated in culture. These results were strongly supported by Maqsood et al. (2012) who suggested that different concentrations of sodium alginate and $CaCl_2 \cdot 2H_2O$ would not only determine the shape of the formed synthetic seeds but also dynamically influence their germination potential.

In consequent to germination, some of the encapsulated SEs were observed to generate beige-colored secondary calluses (Fig. 1d), while some other promoted secondary calluses with translucent and fairly defined SSEs (Fig. 1e). Encapsulated SSEs in optimum encapsulation matrix (3.0% and 100 mM $CaCl_2 \cdot 2H_2O$) as synthetic seeds were later observed to produce multiplied SSEs after another 4–8 weeks on similar culture medium and condition. Transfer of the germinated SSEs on MS medium containing 1.0 mg/L BAP and 1.0 mg/L NAA under continuous light condition at 25 ± 2 °C and 16/8 h photoperiod promoted elongation of SSEs into translucent microshoots (Fig. 1f) within 2 weeks of culture, which later slowly developed green pigments (chlorophyll) as a sign of active photosynthesis activity. The highest number of shoot regeneration at 7.75 ± 1.32 was eventually observed and recorded from the synthetic seeds developed with the optimum encapsulation matrix treatment (3.0% and 100 mM $CaCl_2 \cdot 2H_2O$) after 12 weeks (Table 4). Developed shoots with normal morphology were later photographed (Fig. 2). Root regeneration was unavailable in all treatments tested.

Table 4 The shoot and root regeneration of the germinated synthetic seeds formed with different concentrations of sodium alginate and $CaCl_2 \cdot 2H_2O$ inoculated on MS + 1.0 mg/L BAP + 1.0 mg/L NAA after 12 weeks

Sodium alginate (%)	$CaCl_2 \cdot 2H_2O$ (mM)	12 weeks on MS + 1/0 mg/L BAP + 1.0 mg/L NAA	
		No. of shoots (mean ± SE)	No of roots (mean ± SE)
1.0	50	$0.00 \pm 0.00_a$	$0.00 \pm 0.00_a$
	75	$0.00 \pm 0.00_a$	$0.00 \pm 0.00_a$
	100	$0.00 \pm 0.00_a$	$0.00 \pm 0.00_a$
2.0	50	$1.00 \pm 0.58_a$	$0.00 \pm 0.00_a$
	75	$0.00 \pm 0.00_a$	$0.00 \pm 0.00_a$
	100	$4.50 \pm 0.29_b$	$0.00 \pm 0.00_a$
3.0	50	$0.00 \pm 0.00_a$	$0.00 \pm 0.00_a$
	75	$0.00 \pm 0.00_a$	$0.00 \pm 0.00_a$
	100	$7.75 \pm 1.32_c$	$0.00 \pm 0.00_a$
4.0	50	$0.00 \pm 0.00_a$	$0.00 \pm 0.00_a$
	75	$0.00 \pm 0.00_a$	$0.00 \pm 0.00_a$
	100	$0.00 \pm 0.00_a$	$0.00 \pm 0.00_a$

Values followed by the same letter(s) within a column are significantly different by Tukey-test at $P < 0.05$. SE standard error ($n = 4$)

In terms of storage, it was determined that incubated synthetic seeds at the temperature of 4 °C promoted consistent declination rate of germination within 0–120 days when cultured on basal MS media. The highest germination rate was recorded at 80–40% within 0–15 days of storage and continuously declining to 10% within 60–75 days. By 90–120 days of storage, all synthetic seeds refrigerated at the mentioned temperature failed to germinate (Table 5). In contrast, non-incubated synthetic seeds and those stored at normal culture environment at 25 °C had promoted an optimum 80% germination rate within 0–45 days of storage. An optimum number of shoot regeneration at 11.00 ± 0.91 and 12.25 ± 1.32 with the average number of root regeneration at 3.00 ± 0.41 were also recorded from the mentioned synthetic seeds cultured on basal MS media. The germination rate started to decline at 70% after 60 days and consistently declining to 20% after 90 days of

Fig. 2 Microshoots evolving into normal shoots with developing chlorophyll (green pigments) after 12 weeks of culture on MS + 1.0 mg/L BAP + 1.0 mg/L NAA under continuous light condition at 25 ± 2 °C and 16/8 h photoperiod

Table 5 The germination response of sugar palm's synthetic seeds refrigerated at 4 °C at 30, 60, 90, and 120 days of storage cultured on MS + 1.0 mg/L BAP + 1.0 mg/L NAA after 8 weeks

Storage temperature	4 °C		
Storage duration (days)	Germination rate (%)	No. of shoots (mean ± SE)	No. of roots (mean ± SE)
0	80	$12.25 \pm 1.32_{b}$	$0.00 \pm 0.00_{a}$
15	40	$1.25 \pm 0.75_{a}$	$0.00 \pm 0.00_{a}$
30	30	$0.00 \pm 0.00_{a}$	$0.00 \pm 0.00_{a}$
45	20	$0.00 \pm 0.00_{a}$	$0.00 \pm 0.00_{a}$
60	10	$0.00 \pm 0.00_{a}$	$0.00 \pm 0.00_{a}$
75	10	$0.00 \pm 0.00_{a}$	$0.00 \pm 0.00_{a}$
90	–	$0.00 \pm 0.00_{a}$	$0.00 \pm 0.00_{a}$
105	–	$0.00 \pm 0.00_{a}$	$0.00 \pm 0.00_{a}$
120	–	$0.00 \pm 0.00_{a}$	$0.00 \pm 0.00_{a}$

Values followed by the same letter(s) within a column are significantly different by Tukey-test at $P < 0.05$. SE standard error ($n = 4$)

Table 6 The germination response of sugar palm's synthetic seeds refrigerated at 25 °C at 30, 60, 90, and 120 days of storage cultured on MS + 1.0 mg/L BAP + 1.0 mg/L NAA after 8 weeks

Storage temperature	25 °C		
Storage duration (days)	Germination rate (%)	No. of shoots (mean ± SE)	No. of roots (mean ± SE)
0	80	$11.00 \pm 0.91_{d}$	$3.00 \pm 0.41_{b}$
15	80	$9.25 \pm 1.11_{d}$	$1.25 \pm 0.95_{a}$
30	80	$9.00 \pm 1.08_{d}$	$0.00 \pm 0.00_{a}$
45	80	$7.25 \pm 0.95_{cd}$	$0.00 \pm 0.00_{a}$
60	70	$5.00 \pm 1.08_{bc}$	$0.00 \pm 0.00_{a}$
75	50	$3.25 \pm 1.03_{ab}$	$0.00 \pm 0.00_{a}$
90	20	$0.00 \pm 0.00_{a}$	$0.00 \pm 0.00_{a}$
105	10	$0.00 \pm 0.00_{a}$	$0.00 \pm 0.00_{a}$
120	–	$0.00 \pm 0.00_{a}$	$0.00 \pm 0.00_{a}$

Values followed by the same letter(s) within a column are significantly different by Tukey-test at $P < 0.05$. SE standard error ($n = 4$)

storage. After 105 days, more than 90% of the synthetic seeds failed to germinate (Table 6). Contradicted result was reported in *Begonia* in which the synthetic seeds stored at 4 °C had promoted 97–100% germination rate from 0 to 60 days of storage and 80% germination was demonstrated at 90 days (Awal et al. 2008). Nevertheless, both reports emphasized declination of germination with extended storage period, in which the condition was explained to possibly occur as a result of oxygen deficiency and rapid hydration of the matrix beads (Swamy et al. 2009).

4 Conclusion

Somatic embryos (SEs) and secondary somatic embryos (SSEs) at different developmental stages were successfully encapsulated to produce synthetic seeds of sugar palm. Encapsulation matrix of 3.0% sodium alginate and 100 mM $CaCl_2 \cdot 2H_2O$ promoted soft, solid, uniform, round-shaped, and translucent synthetic beads. In consequent to in vitro germination of the synthetic beads on basal MS media, beige secondary calluses and multiplied secondary somatic embryos were regenerated. Germinated synthetic seeds transferred to MS + 1.0 mg/L BAP + 1.0 mg/L NAA promoted optimum shoot and root regeneration after 8–12 weeks of culture. Storage of synthetic seeds at 25 °C under dark condition provided optimum germination within 0–45 days. Shoots of normal morphology were observed after 12 weeks of culture on MS + 1.0 mg/L BAP + 1.0 mg/L NAA under light condition.

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Perspectives of Synthetic Seed Technology for Conservation and Mass Propagation of the Medicinal Plant *Castilleja tenuiflora* Benth.

Gabriela Trejo-Tapia, Yatzil León-Romero, Eridiana Beatriz Montoya-Medina, Alma Rosa López-Laredo, and José Luis Trejo-Espino

Abstract The production of synthetic seeds through encapsulation is a technology that is used for the storage and conservation of germplasm of medicinal plants, as well as their propagation. This chapter describes the protocol used for the production of synthetic seeds of *Castilleja tenuiflora* Benth. Different concentrations of sodium alginate and calcium chloride dihydrate had an effect on the shape of the capsules and their transparency and consistency. With 3% sodium alginate and 50 mM calcium chloride dihydrate, the capsules were spherical, translucent, and firm in consistency. On the other hand, the storage of synthetic seeds elaborated with micro-stakes of *C. tenuiflora* at 4 °C and in darkness for a period of 50 days allowed a germination of 86% and a conversion of 90% without modifying the chemical profile of the plants generated. Additionally, the feasibility of generating biomass in in vitro conditions from the use of synthetic seeds is demonstrated.

Keywords *Castilleja tenuiflora* · Synthetic seeds · Medicinal plants

1 Introduction

The production of synthetic seeds by means of encapsulation has received great attention in recent years, because it is an excellent option for the biodiversity conservation and mass propagation of species with ecological, economic, and cultural importance (Sharma et al. 2013). A synthetic seed is a somatic embryo, a shoot, a bud, or any other artificially encapsulated meristematic tissue that can be stored and,

G. Trejo-Tapia · Y. León-Romero · E. B. Montoya-Medina · A. R. López-Laredo · J. L. Trejo-Espino (✉)
Natural Products Laboratory, Biotechnology Department, Centro de Desarrollo de Productos Bióticos, Instituto Politécnico Nacional, Yautepec, Morelos, Mexico
e-mail: jtrejo@ipn.mx

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subsequently, sown simulating a functional seed that has the ability to become a plant under in vitro or ex vitro conditions (Ara et al. 1999). The most used compounds that have given positive results in the encapsulation process of synthetic seeds are sodium alginate (Alg-Na) in complex with calcium chloride dihydrate ($CaCl_2$ $2H_2O$), using different concentrations and polymerisation times. The additional advantage is that these ions are easily managed, nontoxic, and low priced (Redenbaugh et al. 1987).

The conservation of germplasm using synthetic seeds has economic advantages with respect to the conventional plant cell and tissue culture, mainly because subcultures are not necessary within short periods of time. This procedure minimises maintenance costs and diminishes the risks of contamination, and the space required for storage is considerably reduced. In addition, synthetic seeds are easy to transport, which facilitates the exchange of germplasm (Sharma et al. 2013).

One of the groups of plants that are being extensively used for the application of this technology for conservation purposes are medicinal plants. A large number of these species are wild, and their seeds are fragile, sensitive to drying, and recalcitrant or feature low viability (Gantait et al. 2015). Some of the most recent reports addressing the production of synthetic seeds of medicinal plants are found in the studies conducted by Gopala Sundararaj et al. (2010). These authors developed a protocol for the production of synthetic seeds using micro-buds from *Zingiber officinale* Rosc. On the other hand, Dhir and Shekhawat (2013) encapsulated nodal segments from in vitro cultures of *Ceropegia bulbosa* in order to promote large-scale mass propagation, short-term conservation, and exchange and distribution of germplasm. Prasad et al. (2014) describe an efficient method for the preparation, short-term conservation, and regeneration of seedlings from synthetic seeds of *Centella asiatica* (L.) Urban. Saeed et al. (2018) developed synthetic seeds from nodal segments of *Gymnema sylvestre* with the purpose of exploiting the encapsulation technology to achieve high recovery of seedlings, short-term storage, and plant conservation.

Castilleja tenuiflora Benth. (Orobanchaceae) is a hemiparasitic and herbaceous perennial or sub-shrubby plant, with highly branched erect stems reaching 30 cm to 1 m. It features racemose inflorescences with acute apex and colours ranging from yellow to reddish orange. This plant grows wild in the highlands of Mexico (between 1300 and 3500 metres above sea level), primarily found in temperate pine-oak forests, scrubs, and grasslands. It is also commonly found both on the edges of agricultural fields and roadsides (Rzedowski and Rzedowski 2005).

Castilleja tenuiflora has a cultural and ethnobotanical importance in Mexico, because, since the sixteenth century, it has been used as a medicinal plant to treat conditions such as cough, dysentery, nerves, menstrual pain and inflammation, gastrointestinal diseases, and tumours (Hernández 1943). Its medicinal properties are attributed to the biological activities that have been proven for this species. It has been reported that wild plants and cultivated in vitro have anti-inflammatory and anti-ulcerogenic activity (Sánchez et al. 2013; Carrillo-Ocampo et al. 2013). It also has cytotoxic effects (Nguyen et al. 2005), antioxidants (López-Laredo et al. 2012), and antidepressants (Herrera-Ruiz et al. 2015). These biological activities have been related to the secondary metabolites present in wild plants and cultivated in vitro. These compounds are iridoid glycosides (aucubin and bartsioside) (Martínez-Bonfil

et al. 2011), phenylethanoid glycosides (verbascosides and isoverbascosides) (Gómez-Aguirre et al. 2012), flavonoids (apigenin and quercetin) (López-Laredo et al. 2012), and lignans (tenuifloroside) (Herrera-Ruiz et al. 2015).

Castilleja tenuiflora is extracted from its natural environment and commercialised in markets of medicinal plants. This overexploitation, coupled with the degradation of its habitat and the fragility of its seeds, may put the permanence of the species at risk. In order to conserve it, efforts have been made to develop biotechnological systems for its mass propagation (Salcedo-Morales et al. 2009; Martínez-Bonfil et al. 2011) and obtain bioactive compounds (Valdez Tapia et al. 2014; Gómez-Aguirre et al. 2012; Medina-Pérez et al. 2015). These systems based on in vitro culture make biomass available for use. However, the technology of synthetic seeds is a method that allows increasing subculture periods and storage time, offering a system for the conservation of germplasm. We describe the protocol that is used for the production of synthetic seeds of *C. tenuiflora*, the effects of the concentration of the encapsulating agents used, their short-term storage, and the advances in the mass propagation of the plant using this technology.

2 Production of Synthetic Seeds of *Castilleja tenuiflora*

Plants of *C. tenuiflora* cultivated in vitro were used as sources of explants for the production of seeds (Salcedo-Morales et al. 2009). The plants were maintained by means of subcultures on Murashige and Skoog (MS) medium (1962), supplemented with 3% sucrose, 0.1 mg L^{-1} of indole-3-butyric acid (IBA), 0.25 mg L^{-1} of 6-benzylaminopurine (BAP), and 0.8% agar, and the pH was adjusted to 5.8 before autoclaving. They were incubated in a culture room with a controlled temperature of $25 \pm 2\,^{\circ}C$, light intensity of 77 μmol m^{-2} s^{-2}, and photoperiod of 16 h of light and 8 h of darkness. The explants used were micro-stakes (segments of stem with two nodes counted from the base of the stem) (Fig. 1a) of 5 mm in length obtained from *C. tenuiflora* plants (mother plants) cultivated in vitro for 35 days.

For producing the synthetic seeds, we prepared two solutions separately, one containing 3% Alg-Na in MS medium (described above) and 100 mM of $CaCl_2$ $2H_2O$ in sterile distilled water. Subsequently, under aseptic conditions, the micro-stakes were added to the Alg-Na solution to be coated with it. Then, with the help of a micropipette (with the disposable tip adapted to have 7 mm in diameter and obtain a homogeneous droplet size), we aspirated explant by explant together with Alg-Na and poured one by one in the form of drops in the $CaCl_2$ $2H_2O$ solution, where they were kept under orbital agitation at 100 rpm for 25 min to promote the adequate formation of the capsule. Once the polymerisation time had elapsed, the synthetic seeds (Fig. 1b) were recovered by decantation, subjected to three consecutive washes with sterile distilled water, and allowed to dry for 1 min on filter paper under aseptic conditions.

With this protocol, it is possible to obtain synthetic seeds of *C. tenuiflora* of 6–7 mm in diameter (Fig. 1b). However, it is important to determine the concentrations of Alg-Na and $CaCl_2$ $2H_2O$ suitable to achieve an effective gelification process

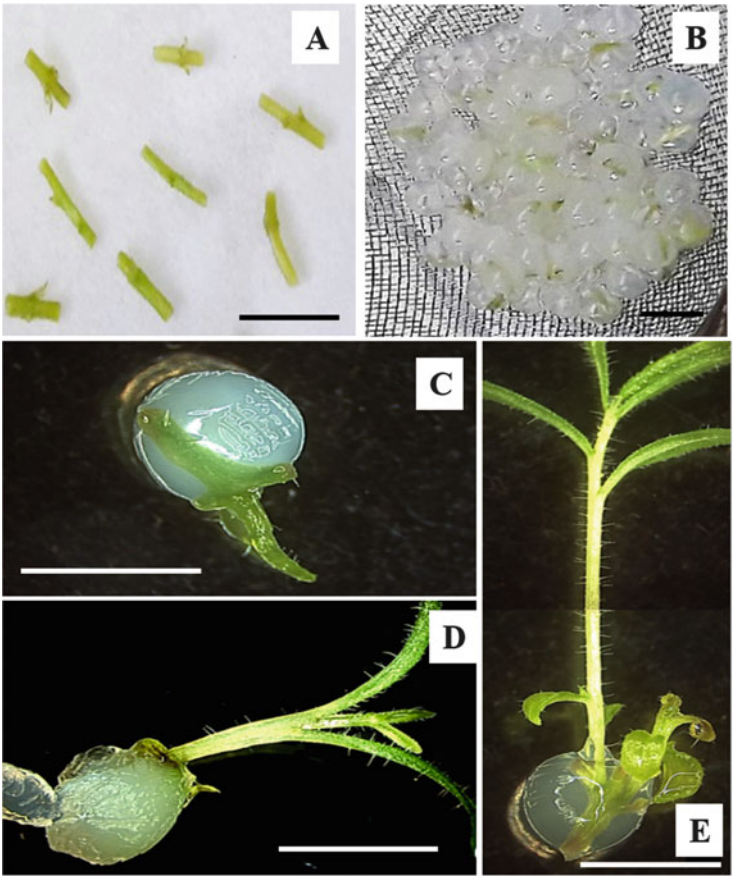

Fig. 1 Procedure for the production of synthetic seeds of *Castilleja tenuiflora.* (**a**) Explants (micro-stakes) from in vitro plantlets. (**b**) Synthetic seeds in Alg-Na. (**c**) Shoot emergence (germination). (**d**, **e**) Micro-stakes conversion to plantlet. Bar = 5 mm

of the matrix and, consequently, optimum quality of the Alg-Na beads that allow the storage of the seeds and promote successful mass propagation of the plants (Dhir and Shekhawat 2013).

3 Selection of Alg-Na and $CaCl_2.2H_2O$ Concentrations

Even though there are reports according to which optimum concentrations of Alg-Na and $CaCl_2$ $2H_2O$ have been found for the production of synthetic seeds of different medicinal plant species, it is necessary to define these concentrations for each species, type of explant, and system used for seed production (Gantait et al. 2015).

In order to determine the concentrations of Alg-Na and $CaCl_2$ $2H_2O$ to be used, we tested three concentrations of both Alg-Na (2, 3, and 4%) and $CaCl_2$ $2H_2O$ (50, 75, and 100 mM), with a polymerisation time of 25 min. After processing, the seeds were placed to germinate in 10 cm long glass tubes with 1 cm in diameter (one seed per tube, 30 tubes per treatment), containing 1.5 ml of MS medium with 3% sucrose and phytagel (15 gL^{-1}). They were incubated in the culture room under the conditions described above. The percentages of germination (seeds that broke the capsule, Fig. 1c) and conversion (seeds that became seedlings, Fig. 1d, e) were measured after 3 weeks of incubation.

Additionally, we measured the resistance of the seeds using a digital force gauge (Chatillon® DFIS series). Force was applied on the seeds produced with the different concentrations of Alg-Na and $CaCl_2$ $2H_2O$ using the flat geometry of the equipment, and the force (N) needed to break them was recorded. Also, observations of the synthetic seeds were made using an environmental scanning electron microscope (Carl Zeiss EVO LS10, Germany). The samples were placed on an aluminium tray with double-sided carbon tape and observed directly. All observations were carried out under constant operating conditions, i.e. 20 kV beam acceleration voltage, using a retrograde electron detector and a water pressure of 30 Pa. The images were captured with 2048 × 1536 pixels and converted into TIFF format.

The results indicated that the concentrations of Alg-Na and $CaCl_2$ $2H_2O$ had different effects on the physical characteristics of the seeds and on the percentages of germination and conversion processes. With low concentrations of Alg-Na (2%) and $CaCl_2$ $2H_2O$ (50 mM), we obtained soft and fragile capsules without a defined shape (Fig. 2a, d), which caused difficulties in handling. For this reason, it was not possible to measure the necessary force to break the capsules in this group of seeds or subject them to the germination and conversion test. With intermediate concentrations of Alg-Na (3%) and low and intermediate concentrations of $CaCl_2$ $2H_2O$ (50 mM and 75 mM), it was possible to obtain spherical seeds, with a translucent appearance and adequate firmness for handling (Fig. 2b, e). There was no significant difference between these treatments, and the percentages obtained were 60 and 63% for germination and 86 and 90% for conversion, respectively.

When the seeds were submitted to the resistance test, it was necessary to apply a force ranging from 5.7 N to 6.3 N to deform and break the capsules. In contrast, with higher concentrations of both Alg-Na (4%) and $CaCl_2$ $2H_2O$ (100 mM), spherical and opaque seeds with firm consistency were produced (Fig. 2c, f). However, the germination and conversion percentages decreased to 40 and 60%, respectively, whereas the force needed to break the seeds increased to values between 9.5 N and 10.3 N. These results are in line with those reported by Dhir and Shekhawat (2013) for *Ceropegia bulbosa*, according to which the concentration of the encapsulating agents was determinant for the adequate formation of the capsules, and the results reported by Singh et al. (2010), who found that the synthetic seeds of *Eclipta alba* produced with different concentrations of Alg-Na and $CaCl_2$ $2H_2O$ had morphological differences in terms of texture, shape, and transparency.

In addition to the adequate physical characteristics of the capsules, it is also important that they allow their storage and maintain the viability of the seeds in terms

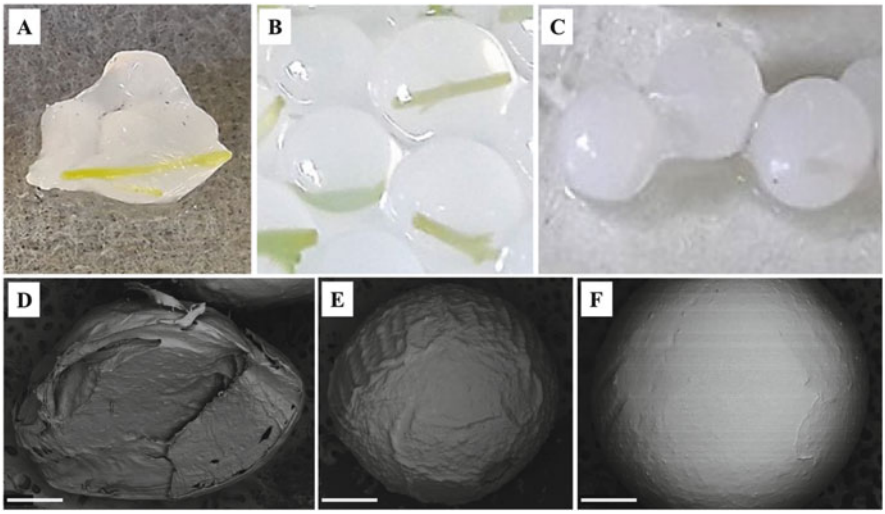

Fig. 2 Synthetic seeds of *Castilleja tenuiflora* obtained with different concentrations of Alg-Na and $CaCl_2$ $2H_2O$. (**a**, **d**) Seeds made with 2% Alg-Na + 50 mM $CaCl_2$ $2H_2O$. (**b**, **e**) Seeds made with 3% Alg-Na + 50 mM $CaCl_2$ $2H_2O$. (**c**, **f**) Seeds made with 4% Alg-Na + 100 mM $CaCl_2$ $2H_2O$. (**d**, **e**) Environmental scanning electron micrographs (beam acceleration voltage of 20 kV with a backscattered electron detector and water vapor pressure of 30 Pa). Bar = 5 mm

of re-growth and conversion (Gantait et al. 2015). From the results found, the concentrations selected to produce the synthetic seeds of *C. tenuiflora* were 3% of Alg-Na and 50 mM of $CaCl_2$ $2H_2O$. These conditions enable the capsules to protect the explants and avoid mechanical damages. At the same time, the explants can break the capsules at the time of germination and plant conversion can take place.

4 Short-Term Preservation of Synthetic Seeds of *Castilleja tenuiflora*

In order to determine the appropriate storage temperature, the synthetic seeds of *C. tenuiflora* were stored in cryotubes (10 seeds per cryotube, 3 cryotubes per temperature) for 45 days, under dark conditions at three different temperatures (1.5, 4, and 25 °C). After this period, the viability of the seeds was assessed according to what was reported by Micheli et al. (2007), who affirmed that a synthetic seed is viable if the explant maintains a green colour, without necrosis or areas with yellow colouration.

All the seeds stored at 4 °C remained viable during the 45 days of storage, whereas those stored at 1.5 and 25 °C exhibited a viability of 70 and 76%, respectively. There were seeds with necrotic areas and tissue oxidation. Based on these results, two groups of 30 seeds were placed into 5 ml cryotubes under dark conditions, at 4 °C, and

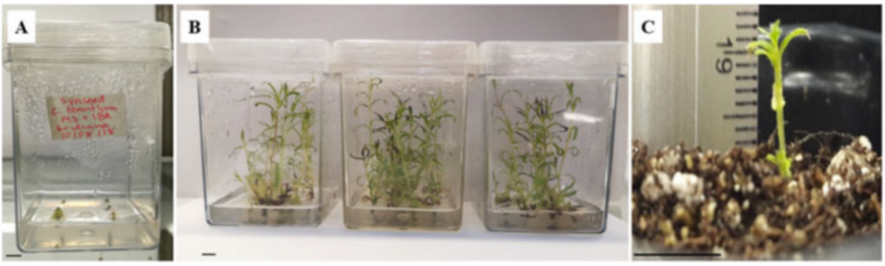

Fig. 3 *Castilleja tenuiflora* plantlets propagated from synthetic seeds maintained at 4 °C for 50 days. (**a**) Synthetic seeds. (**b**) *Castilleja tenuiflora* plantlets propagated in vitro from synthetic seeds. (**c**) *Castilleja tenuiflora* plant developed from synthetic seeds grown in mix pot. Bar = 5 mm

stored for 50 days. After this storage time, the seeds were placed into magenta boxes (Fig. 3a) with MS medium plus 3% sucrose, supplemented with 7 μM of IBA. As control, we sowed synthetic seeds which had not been stored, and all the seeds were incubated in a culture room with controlled temperature (25 ± 2 °C), light intensity of 77 μmol m^{-2} s^{-2}, and photoperiod of 16 h of light and 8 h of darkness. After 3 weeks, we assessed the percentages of germination and conversion and observed whether the plants had sprouts or not.

The effect of storage at 4 °C under dark condition was positive for the viability of the seeds in terms of re-growth, reaching a germination of 77% and a conversion of 70%. These results were significantly different from the results obtained from the control group (synthetic seeds without storage), which only reached values of 30% and 20%, respectively. Kamińska et al. (2018) reported that storage of synthetic seeds of *Taraxacum pieninicum* at 4 °C under dark conditions promoted an increase in their conversion capacity. It is possible that this response is related to the low ABA/GA ratio present under dark conditions, which leads to an increase in the survival and proliferation of plants originated from stored seeds.

In addition, cold storage is the simplest and most efficient method to reduce in vitro growth (Kamińska et al. 2018). Ray and Bhattacharya (2010) obtained high conversion percentages (82.4%) when they stored synthetic seeds of *Eclipta alba* at 4 °C. At low temperatures, synthetic seeds acquire a state of dormancy in which metabolic activities are reduced, allowing them to be stored for different periods of time. The explants retain the intrinsic nutrients and can germinate and become plants when placed under optimal conditions.

5 Mass Propagation of *Castilleja tenuiflora* Obtained from Synthetic Seeds

Synthetic seeds stored for 50 days under dark conditions and at 4 °C were placed to germinate in magenta boxes with MS culture plus 3% sucrose, supplemented with 7 μM of IBA. After 3 weeks, the synthetic seeds germinated and formed leaf primordia, seedlings, or plants that were used to perform two independent tests for

their mass propagation. In the first, the plants were used as a source of explant (nodal segments) and placed in magenta boxes with fresh culture medium under aseptic conditions (Fig. 3a). In the second test, the seedlings were transplanted directly into pots, with mixed substrate (peat/agrolite/vermiculite, 60:20:20) under non-aseptic conditions. Both assays were incubated in a culture room under controlled conditions (previously described).

The results of the first trial demonstrated the feasibility of using synthetic seeds for mass propagation and in vitro conservation of *C. tenuiflora*. The percentage of explants that produced shoots was 100%, with seven shoots per explant. This meant an average production of 40 plants with a length ranging from 7 to 8 cm from each synthetic seed after 3 weeks of cultivation (Fig. 3b). This protocol allows providing a constant source of plant material and could help to avoid the overexploitation of wild plants, with the advantage that synthetic seeds also allow the storage of germplasm and its subsequent conservation.

The plants obtained from synthetic seeds that developed in mixed substrate were able to grow and form root after 30 days of cultivation (Fig. 3c). However, their size was eight times less than those developed in a semi-solid medium. This result indicates that the mixed substrate used provided the plants with a support medium, but not with a system containing adequate nutrients, moisture, and porosity for the proper development of the plants. Therefore, we continued conducting studies aimed at testing different types of substrates and their proportions, as well as different incubation conditions that could allow an adequate ex vitro development of the plants obtained from synthetic seeds.

6 Chemical Profile of *Castilleja tenuiflora* Plants Obtained from Synthetic Seeds

In order to know whether the production process and cold storage of synthetic seeds caused a change in the chemical profile of *C. tenuiflora* or not, we prepared methanolic extracts from plants grown from synthetic seeds obtained in storage tests at 4 °C for 50 days and plants cultivated in vitro (mother plants) used as a source of explants for the production of seeds. To prepare the extract, the dried samples were placed in 2.5 ml Eppendorf tubes and ground in the tissue homogenizer (TissueLyser) for 5 min at 100 rpm. Subsequently, the sample was suspended in a 5 ml tube, with 1.5 ml of methanol (1:50 ratio), and left in a sonicator apparatus for 1 h at room temperature. The methanolic extract was filtered using Whatman No. 1 filter paper and allowed to evaporate to dryness. The extracts were stored under dark conditions until being analysed.

We used the chromatographic method proposed by Cortes-Morales et al. (2018) for the analysis of the extract, with an LC-MS 2020 equipment (Shimadzu, Tokyo, Japan), a CBM-20A system controller, two LC-20 AD pumps, a DGU-20A 5R degassing unit, a SIL-20 AC HT Autosampler, a CTO 20A column oven, and an SPD-M20A photodiode array detector (PAD). We performed a high-resolution

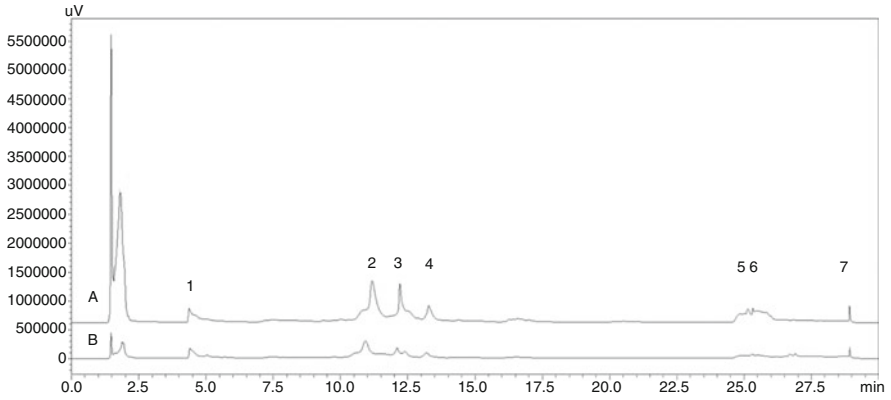

Fig. 4 Chromatogram of the chemical profile of *Castilleja tenuiflora* plantlets. (A) In vitro plantlets (control). (B) Plantlets developed from synthetic seeds maintained for 50 days at 4 °C. Peak 1 (t_R = 04.54) corresponded to aucubin, peak 2 (t_R = 11.28) corresponded to isoverbascoside, peak 3 (t_R = 12.25) was a phenylethanoid, peak 4 (t_R = 13.43) corresponded to verbascoside, and peaks 5, 6, and 7 corresponded to lignan-type phenolic compounds

global analysis of liquid chromatography coupled to a simple quadrupole mass spectrometer (HPLC-MS). The major chemical compounds were identified by their wavelength (λ), retention time (t_R), and total ion count (m/z).

The comparison of the chemical profiles obtained by HPLC is illustrated in Fig. 4. The results indicated that there was no difference between the chemical compounds present in the mother plants (control) (profile A) and those obtained from synthetic seeds stored for 50 days under dark conditions and at 4 °C (profile B). The compounds corresponded to the groups already reported for wild plants and cultivated in vitro, i.e. glycosylated iridoids (Martínez-Bonfil et al. 2011, glycosylated phenylethanoids (Gómez-Aguirre et al. 2012), flavonoids (López-Laredo et al. 2012), and lignans (Herrera-Ruiz et al. 2015). Peak 1 (t_R = 04.54) corresponded to aucubin, peak 2 (t_R = 11.28) corresponded to isoverbascoside, peak 3 (t_R = 12.25) was a phenylethanoid, peak 4 (t_R = 13.43) corresponded to verbascoside, and peaks 5, 6, and 7 corresponded to lignan-type phenolic compounds. These results confirmed that secondary metabolism was not affected in terms of the biosynthesis of chemical compounds of *C. tenuiflora* when subjected to the encapsulation process and storage at low temperatures.

7 Conclusions

The technology of synthetic seeds applied to the conservation and clonal propagation of plant species with ethnobotanical and economic importance, threatened or endangered, is a promising, economical, and feasible method. The protocol for the

production of synthetic seeds and storage at 4 °C, described in this work, allows the conservation of germplasm in the short term and the subsequent in vitro mass propagation of *C. tenuiflora*, without losing the medicinal characteristics of the plant due to the fact that the process does not promote changes at the level of its chemical profile. However, it is necessary to conduct further studies with longer storage times in order to obtain information about aspects of ex vitro mass propagation using synthetic seeds and the genetic stability of this species.

This is the first report on the use of synthetic seeds of *C. tenuiflora*. It can also serve as a basis for the implementation of the process using other threatened species belonging to the plant family Orobanchaceae.

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Encapsulation and Synthetic Seeds of Olive (*Olea europaea* L.): Experiences and Overview

Maurizio Micheli, Alvaro Standardi, and Daniel Fernandes da Silva

Abstract Olive (*Olea europaea* L.) is one of the species more representative of Mediterranean basin where it is strictly connected to the history and culture of the local populations. In the last 20 years, the olive tree diffusion has increased out of traditional growing area, due to the appreciated value of olive oil and its properties. So, a greater amount of plants has been requested to satisfy the worldwide demand. Commercialization of high-quality plant material could be simplified by using the encapsulation technology products, as beads (or capsules) and synthetic seeds (or synseeds or artificial seeds). State of the art on the encapsulation of olive in vitro-derived microcuttings is discussed. Firstly they were used to prepare beads able to develop new shoots also after storage; in fact the capsules make easier the exchange of plant material between laboratories and countries. Moreover, encapsulated microcuttings could be employed to prepare synthetic seeds from which plantlets arise after sowing in greenhouse. But in olive these biotechnological tools are still under investigation.

Keywords Microcutting · cv. Moraiolo · Alginate matrix · Artificial endosperm · Bead · Inductive treatment · Cold storage · Regrowth · Conversion

1 Introduction

Olive (*Olea europaea* L.) is traditionally one of the most important fruit crops of Mediterranean area. This species is mainly cultivated for production of fine oil, always more valued worldwide and appreciated from an increasing number of

M. Micheli (✉) · A. Standardi
Department of Agricultural, Food and Environmental Sciences (DSA3), University of Perugia, Perugia, Italy
e-mail: maurizio.micheli@unipg.it

D. Fernandes da Silva
Centro de Ciencias Agrarias, Western Paranà State University (Unioeste), Marechal Candido Rondon, Paranà, Brasil

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consumers. In the last two decades, the cultivation has expanded to other regions, as Asia, Oceania, and South America, with great commercial perspectives for traditional olive plant producers. According to data published by the International Olive Council (IOC), the world's olive-growing area reached approximately 11.4 million ha in 2015 (Rallo et al. 2018), and the total production of olive fruits is about 19 million tons, of which 10% are used as table olives and 90% are processed to obtain olive oil (Petruccelli and Ganino 2018). Since the requirement of plantlets for new orchards is quite high and increasing, the conventional techniques of propagation by seeds or asexual methods by grafting and cutting seem not able to satisfy the olive plant market. New methods of in vitro propagation could be supplementary or alternative tools for nursery activity. Micropropagation allows mass productions of valuable genotypes in shorter periods independently of seasonal issues because it is carried out under controlled environment. But commercial application remains still limited in olive, and plant tissue culture studies are restricted because of specific procedural problems. In addition, large-scale deployment of micropropagated plantlets can implicate difficulties on management, storage, or transport and easily exposes them to deterioration and damage risks (Standardi and Micheli 2013).

New in vitro technologies are being studied to simplify the exchange of plant material between producer and final user laboratories/nurseries, especially from different countries where intransigent regulations are applied to prevent introduction of pathogens. Encapsulation seems to be a promising technology in the service of nursery operators and farmers. In olive the production of explants usable for encapsulation is relatively easy by the micropropagation. After that in vitro-derived propagules can be covered by a layer of calcium alginate matrix enriched with an artificial endosperm which assures protective and nutritive functions. Two can be the final products: capsules or beads and synthetic (or artificial) seeds or synseeds. The first ones can be defined as encapsulated portions of in vitro-derived plant tissues possessing the ability to evolve in shoots (regrowth), which can be reused only for micropropagation after storage and/or transport. Synthetic seeds instead are able to develop whole plantlets (conversion) under in vitro or ex vitro conditions (Standardi and Micheli 2013). Some years ago Sharma and coworkers (Sharma et al. 2013) affirmed that the encapsulation is really applicable for germplasm conservation and exchange of valuable rare hybrids and elite genotypes. Recent studies reported the use of beads and synthetic seeds also to simplify the management of mass clonal propagation products in different species (Ravi and Anand 2012; Reddy et al. 2012; Gantait and Kundu 2017; Magray et al. 2017). Encapsulation of olive plant material is still not largely studied as the fruits or the medicinal plants in the face of the great interest showed toward this species.

2 Experiences in Olive

The history of encapsulation begins from the original concept of synthetic seeds highly connected with the use of somatic embryos able to develop a whole plantlet (conversion) (Murashige 1978). That makes them the ideal explants to be

encapsulated. But the difficulty to set effective protocols to achieve somatic embryos in a great number of species and the risks of somaclonal variations during in vitro embryogenesis puts serious problems for application in the nursery activity (Standardi and Micheli 2013). Later Bapat and collaborators (Bapat et al. 1987) proposed to broaden this technology to different in vitro-derived propagules until a new definition of synthetic seed was formulated as "artificially encapsulated somatic embryos, shoots, or other tissues which can be used for sowing under in vitro or ex vitro conditions" (Aitken-Christie et al. 1995). As reported by Lambardi et al. (2006), "new" synthetic seeds can be prepared by any kind of in vitro-derived explants as axillary buds, shoot tips, nodal segments, bulblets, protocorms, and callus samples or cells, as like as they evolve in plantlets after encapsulation and sowing.

Our researches on olive were focused on the use of non-embryogenic microcuttings (3–4-mm-long nodal portions with apical or axillary buds), excised from micropropagated shoots, genetically stable, but naturally unable to develop whole plantlets without rooting inductive treatments (Standardi and Micheli 2013; Sharma et al. 2013; Gantait et al. 2015; Rihan et al. 2017).

We specifically worked on olive which is quite difficult to be micropropagated, even though the technique shows future high potentialities about its commercial application for production of high-quality plants. So, we will describe the path done to develop synthetic seeds of olive; it was long, difficult, and complicated due to the limitation above mentioned. Therefore, below the key points of our salient experiments conducted to produce synthetic seeds by using microcuttings will be described in detail. In particular our researches involved the cv. Moraiolo, typical genotype of Central Italy and required from farmers of some countries producing high-quality oil.

The experiments were divided into two main aspects: (a) the initial studies were focused on the detection of encapsulable non-embryogenic propagules of olive and on the factors affecting their performances and (b) the following researches were aimed to induce rooting ability in unipolar microcuttings in order to obtain synthetic seeds.

2.1 Selection of Explants

Preliminarily, the appropriate size of encapsulable in vitro-derived explants was studied. Therefore, the sprouting ability of axillary microcuttings and 10–15-mm-long minicuttings excised from in vitro-derived shoots was monitored in absence of encapsulation. After 45 days of culture on half-strength agarized Olive Medium Modified (OMM) (Mencuccini et al. 1997), no malformation, yellowing, or browning of explants was observed, but their size had a definite effect on the viability (propagules showing green appearance, with no necrosis or yellowing), regrowth (propagules showing development of the shoot), and shoot length (length of the shoots developed from each encapsulated microcuttings). Microcuttings showed viability and regrowth, respectively, less than 31 and 33% in comparison to those monitored in minicuttings (Micheli and Standardi 2005). But the minicuttings are

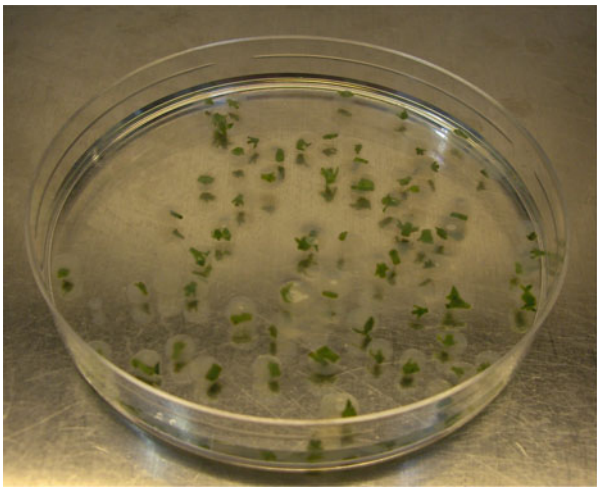

Fig. 1 Encapsulated microcuttings (beads) of 'Moraiolo'

inappropriate because produce long and narrow beads with propagules not perfectly enclosed within the alginate matrix, which loses its protective and nutritive functions (Redenbaugh et al. 1987; Piccioni and Standardi 1995).

The next goal was to individuate the type of microcuttings in connection with their original position on the micropropagated shoots. For this purpose apical and axillary nodal portions 3–4 mm long were isolated and encapsulated as reported by Micheli et al. (1998); the microcuttings were then immersed by forceps in sodium alginate solution 2.5% (encapsulation matrix) and dropped in 11 g 1^{-1} $CaCl_2$ (complexing solution) for 25–30 min. Both complexing and encapsulating chemicals were dissolved in half-strength OMM solution without agar (artificial endosperm). The obtained capsules were rinsed twice for 10 min to wash away chloride and sodium residues. The beads of 'Moraiolo' (Fig. 1) were sown on agarized hormone-free OMM, and after 45 days into growth room, their vegetative performances were monitored evaluating viability, regrowth, and shoot length. The results confirmed the differences between two types of propagules showing higher viability and regrowth of apical microcuttings (80.0%) compared to axillary ones (28.1%). Moreover, the beads containing the apical buds developed longer shoots (12.6 mm against 4.2 mm of those grown from axillary microcutting) (Micheli et al. 1998). The different performances between two types of encapsulated explants could be influenced also by the effect of apical dominance during the proliferation of micropropagated shoots (Rugini and Panelli 1993; Mendoza-de Gyves et al. 2008; Micheli et al. 2018). Nevertheless, the use of apical microcuttings is inappropriate in the perspective to apply the encapsulation technology for the nursery mass productions. For this reason, further studies were carried out using axillary microcuttings only.

2.2 Viability and Regrowth

The initial studies clearly showed that the viability and the regrowth of microcuttings are affected by bud dormancy and low vegetative vigor which cause the limited sprouting ability and the difficulty to break the alginate matrix after sowing (mechanical obstacle). Experiments were carried out to increase regrowth of encapsulated olive microcuttings improving the nutritive function of artificial endosperm and breaking bud dormancy by chemical or physical treatments.

2.2.1 Artificial Endosperm

It is well known that artificial endosperm plays an essential role as trophic source for microcuttings to maintain viability, to stimulate regrowth, and to influence the shoot length. Addition of nutrients and plant growth regulators to encapsulation solutions increased vegetative vigor in encapsulated plant material (Kikowska and Thiem 2011). In this regard, it is well known that in olive micropropagation, cytokinins are an important factor to promote shoot formation and the effectiveness of zeatin is commonly stated (Micheli et al. 2018; Rugini 1990; Grigoriadou et al. 2002; Rugini and Baldoni 2004). This growth regulator is required in high concentrations and is very expensive (Mendoza-de Gyves et al. 2008).

On this basis, some experiments were conducted to evaluate the effect of zeatin on the encapsulated microcuttings of 'Moraiolo' when added (1.0 mg l^{-1}) to the artificial endosperm. The results showed that it didn't determine a real improvement of viability and regrowth while stimulated the elongation of developed shoots which reached 8.1 mm. A clear improvement was achieved when the concentration of sucrose within the artificial endosperm was increased up to 50 g l^{-1}. In this condition, viability and regrowth showed the highest values (90–100%), and the mean of shoot length was 45.7 mm (Fig. 2). The availability of a suitable quantity of sucrose was essential to enhance the encapsulated microcuttings' performances confirming previous results (Gardi et al. 1999; Micheli et al. 2007).

Jung and coworkers (Jung et al. 2004) already described the effectiveness of sugar as component of alginate matrix on conversion of encapsulated somatic embryos of *Siberian ginseng*. Carbohydrates are essential as a source of carbon for biosynthesis energy and have a stabilizing osmotic effect (Rihan et al. 2017). Moreover these substances are of prime importance for cell growth maintenance and in vitro differentiation (Kumara Swamy et al. 2010). Some fundamental aspects of carbon utilization and metabolism in cell and tissue cultures have yet to be fully understood (Srivastava et al. 2017).

Fig. 2 Shoots regrown from beads of olive with residue of alginate matrix

2.2.2 Role of GA_3

Carbohydrates are essential for encapsulated microcuttings of olive also because they interact with hormones playing a stimulatory effect on regrowth. Studies on in vitro culture reported that zeatin and other cytokinins added to culture medium may enhance morphogenesis (shoot initiation/bud formation) in olive, while auxins promote root initiation inducing both growth of pre-existing roots and adventitious root formation. The gibberellins generally regulate growth, stimulate stem elongation, and break dormancy of buds (Niaz et al. 2014).

In order to overcome the effect of bud dormancy in encapsulated microcuttings of olive, some experiments were conducted with the aim to evaluate the effect of a sprouting inductive treatment using GA_3. For this purpose microcuttings of 'Moraiolo' were dipped in 1 mg l^{-1} GA_3 and 30 g l^{-1} sucrose solution under darkness at 23 ± 2 °C and maintained on a rotary shaker (100 rpm) for 24 h. After that microcuttings were placed on a sterile filter paper (Whatman #1) layered on agar medium (half-strength OMM) inside Petri dishes and kept in the growth chamber under darkness for 6 days. Microcuttings were then encapsulated and transferred in growth chamber after sowing on agarized medium. After 45 days of culture, 100% of beads showed viability and regrowth developing shoots from both two axillary buds of the single node and drastically reducing callogenesis up to 0% (Micheli et al. 2007).

These results demonstrated the beneficial effect of GA_3, as reported by Rugini and Fedeli (1990), Cañas et al. (1992), and Menghini et al. (1999), when used as liquid treatment or added to the agarized medium (Dimassi-Theriou 1998; Kotsias and Roussos 2001); GA_3 increased the vigor of explants and promoted the regrowth of buds. In fact, it is well known that gibberellins stimulate the mitotic activity of sub-apical meristems (Machácková 1992; Jacqmard et al. 2003; Pattanagul et al. 2005) and play a

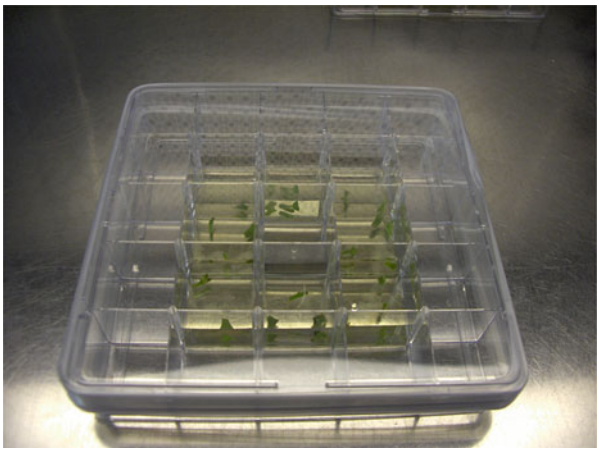

Fig. 3 Beads of 'Moraiolo' stored at 4 °C in 25-squared-well sterile plastic plates

role on breakage of bud dormancy (Augé 1995; Hassani et al. 2014; Zheng et al. 2018). Lastly, absence of callus could also indicate a balanced utilization of the available nutrients (Micheli et al. 2007).

2.2.3 Low-Temperature Treatment

Further experiments were conducted with the aim to evaluate the effect of temperature on the viability and the regrowth of encapsulated microcuttings. For this purpose ten capsules of 'Moraiolo' were placed in single well of 25-squared sterile plastic plates (Fig. 3); each well contained 2 ml of liquid artificial endosperm medium to avoid dehydration. The storage was carried out in dark conditions at 4 °C for 45, 90, 135, and 180 days. At the end of these periods, the beads were sown on agarized hormone-free OMM for 45 days in the growth chamber for viability and regrowth evaluation. The shortest storage period didn't affect viability and regrowth of encapsulated microcuttings (up to 49.0%). In the other storage conditions, total loss of propagules viability was monitored.

These results confirmed that beads can be submitted to cold storage for short-medium period (Piccioni and Standardi 1995) and that the effect of low temperature is strictly dependent on the type of encapsulated explants and genotype (Gardi et al. 1999; Tsvetkov and Hausman 2005; Rai et al. 2008; Parveen and Shahzad 2014; Sharma et al. 2015; Benelli 2016; Hatzilazarou et al. 2018). Cold treatments could be used to break bud dormancy in in vitro cultures as in woody plants in the field (Naor et al. 2003; Caffarra and Donnelly 2011). The drastic decrease in microcuttings viability connected with longer storage period could be attributed to an inhibition of tissue respiration during storage as a result of impeded oxygen diffusion imposed by the alginate matrix (Hatzilazarou et al. 2018).

2.2.4 Combined Effect of GA_3 and Temperature

Further experiments were conducted to study the effect of 1 mg l^{-1} GA_3 in combination with 4 or 18 °C storage temperature on regrowth. After GA_3 application, the microcuttings of 'Moraiolo' were encapsulated and stored for 15 and 30 days in the darkness. Forty-five days after sowing, all beads maintained viability and regrowth irrespective of the storage temperature showing that the combined treatment didn't affect their performances (Micheli et al. 2007). Moreover, the positive effect of room temperature during storage without any loss of viability and regrowth was registered. This can reduce drastically the costs of refrigeration or environment conditioning in the commercial application of encapsulation technology (Ikhlaq et al. 2010).

2.3 Rooting

Usually encapsulated microcuttings are unable to regenerate adventitious roots without specific treatments. In addition, 'Moraiolo' is difficult to root in in vivo conditions too (Bartolozzi 1998), and 90% of olive genotypes show rooting ability lower than 50% (Petruccelli et al. 2012). So, experiments were conducted with the aim to induce rooting ability in microcuttings of this genotype to evaluate the feasibility of synthetic seed formation. In particular the effect of auxin treatments before encapsulation was examined, since its involvement on roots initiation is well documented (Niaz et al. 2014).

De Klerk (2002) proposed that the rooting process in apple microcuttings can be divided into three phases: dedifferentiation (0–24 h from auxin treatment), induction (25–96 h), and differentiation (from 97 h onward). During the first step cells become competent to respond to the hormonal rhizogenic signal; by the action of auxin, certain cells become determined to root formation. After induction, the signal is not required anymore: the determined cells produce roots primordia which initiate growth and emerge from the stem. So, a first experiment was focused to test a rooting procedure in olive microcuttings. They were subjected to a rooting induction (RI), dipping the explants in a 5 mg l^{-1} indole-3-butyric acid (IBA) solution enriched with 15 g l^{-1} sucrose and maintaining them for 24 h in darkness on rotator shaker at 100 rpm. IBA was used because it proved to be a better rooting growth regulator for 'Moraiolo' as compared to other auxins (Niaz et al. 2014). In a second step, before encapsulation and sowing, all microcuttings were placed on a sterile filter paper (Whatman no. 1) previously laid on agarized hormone-free OMM and maintained at darkness for 5 days in growth chamber to induce root primordia initiation (RPI, Fig. 4). The use of filter paper was employed to enhance gas exchanges, as successfully applied in other woody species by Welander and Pawlicki (1993) and Piccioni et al. (1997). After 45 days' culture, in addition to viability and regrowth, rooting (encapsulated propagules showing roots formation)

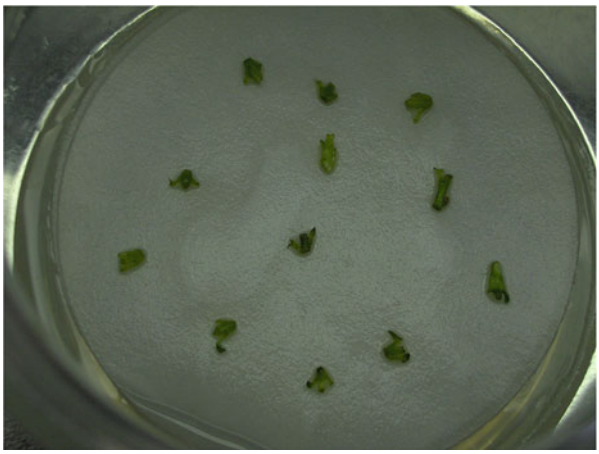

Fig. 4 Microcuttings of olive placed on sterile filter paper during root primordia initiation (RPI) phase

was monitored. The results showed limited viability and regrowth values (respectively, 36.0 and 34.0%) and the total absence of rooting activity.

These preliminary results could be connected with the contemporary presence of zeatin (as component of artificial endosperm) and auxin which notoriously can have antagonistic effects when their ratio is not well balanced, as reported by Caraballo et al. (2010), Wróblewska (2012), and Nakhooda et al. (2012). Our results suggested that cytokinin may reduce the effectiveness of auxin on rooting induction. This could be restored by using higher concentration of IBA or extending the RI duration. Subsequent experiments were carried out increasing to 72 h the RI treatment (Fig. 5, procedure A), as suggested by Capuano et al. (1998) in apple. The results showed 93.0% of viability, 66.7% of regrowth, and 36.7% of conversion demonstrating that the synthetic seeds of 'Moraiolo' were obtained. These results are promising but need further researches because at the moment the conversion is too low for commercial application.

The treatments with exogenous auxin increase the rooting because it is related to endogenous content of the same growth regulator; the accumulation of IBA in the basal region of the vegetative propagules acts as a metabolizing agent and signal to induce rooting (Brondani et al. 2012). Dissecting the rooting process in apple microcuttings, during the first day, certain cells in the stem become competent to respond to the rhizogenic signal. Then, in the next 48 or 72 h under the influence of the root-inducing signal (auxin), the cells divide and the descendent cells become increasingly determined to root formation (De Klerk 2002).

The procedure A (Fig. 5) is laborious and inadequate for application in nurseries. It would be necessary to dispose of semi-automatic/automatic tools to limit hand labor and/or to simplify the procedures in order to reduce costs of synthetic seeds preparation with microcuttings (Standardi and Micheli 2013). Just in this view, further researches were conducted simplifying the auxin treatment. Beads of 'Moraiolo' were directly soaked into the rooting solution for 3 days under darkness conditions at environmental temperature, just before sowing (Fig. 5, procedure B).

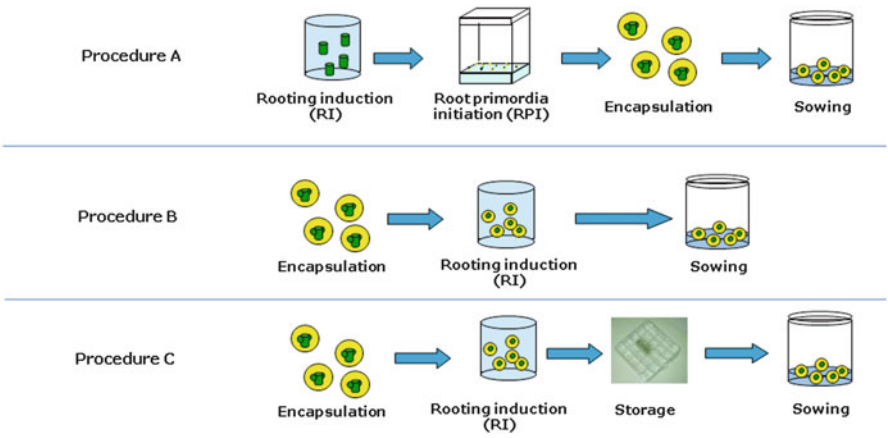

Fig. 5 Synthetic seeds preparation of 'Moraiolo': the conversion was 36.7% from procedure A, 13.0% from B, and 32.7% from C

This methodological shift allowed to optimize viability and regrowth (100%), but the conversion remained clearly unsatisfactory at 13.0% (Micheli et al. 2006).

2.4 Effect of Cold Storage on Conversion

In order to improve the conversion of synthetic seeds of 'Moraiolo' prepared by the simplified procedure B (Fig. 5), further experiments were conducted with the aim to test the effectiveness of a short period storage at low temperatures. For this purpose 7-day cold storage was applied after the rooting induction (Fig. 5, procedure C). After 45 days in the growth chamber, synthetic seeds showed 32.7% of conversion (Fig. 6), confirming the positive effect of cold treatment as reported by Capuano et al. (1998) working with apple rootstock.

Some researchers described the beneficial effects of cold treatment on rooting as Van de Pol (1983) in different species, Nikolić et al. (2008) in *Fritillaria meleagris*, Luo et al. (2009) in protocorm-like bodies of *Dendrobium huoshanense*, and Aktar and Shahzad (2017) in encapsulated nodes of *Glycyrrhiza glabra.*

3 Overview

At the moment the synthetic seeds obtained by encapsulation of in vitro-derived microcuttings of olive (cv. Moraiolo) require high manual labor and showed low conversion levels; so its large application in the nurseries activities is not economic yet.

Fig. 6 Plantlet from synthetic seed of 'Moraiolo'

In some laboratories researches are in progress aimed to increase the conversion response from encapsulated microcuttings by appropriate root inductive treatments which involve exogenous growth regulators and environmental factors as light and temperature.

It was demonstrated that in olive the inclusion of single microcuttings in alginate matrix (capsules) permits to maintain their viability and regrowth until 4 weeks also when stored at room temperature. This knowledge opens a perspective for commercial utilization of the encapsulation technology in combination with the micropropagation one to satisfy the high demand of olive plants for the new orchards expected in some countries. Micropropagated plantlets are inadequate for plant exchange, because they are not easy to manage, to store, and to transport for long distance for related risks of deterioration (Standardi and Micheli 2013). The capsules instead represent the suitable tool for economic store and easy transport of plant material.

So, on the basis of actual knowledge on the encapsulation technology in olive, new approach for international exchange and commercialization could be proposed. In few words, into laboratories of countries in which olive propagation and cultivation are traditional, the capsules should be prepared utilizing microcuttings from shoots proliferated in vitro. Into the greenhouses of the countries of destination, the capsules (beads) would be utilized from local nurseries to obtain shoots for rooting or directly whole plantlets from synthetic seeds, when efficient protocols will be available.

In olive, our findings are surely preliminary and incomplete, but they represent a good point of departure for the next studies aimed to obtain synthetic seeds with satisfactory conversion rate. For this it is essential to pursue (1) widening of genotypic base, (2) optimization of storage techniques, (3) obtaining of conversion in ex vitro conditions, (4) simplification of processes for synthetic seeds production, and (5) introduction of automation/mechanization.

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Somatic Embryogenesis and Synthetic Seed Technology of *Curcuma* spp.

Appakan Shajahan, Chellappan Soundar Raju, Valiyaparambath Musfir Mehaboob, and Abubakker Aslam

Abstract *Curcuma* is a unique spice crop and sterile triploid plant. The rhizomes are used for vegetative propagation and sold for consumption. These rhizomes are highly damaged by biotic and abiotic stress. Alternative approaches of synthetic seed technology will allow the mass propagation for commercial utilization. Execution of this technology requires manipulation of in vitro system for large-scale production of viable propagules. Somatic embryogenesis is the best regeneration system for rapid and true-to-type multiplication of elite and desirable plant species; it offers reduction of time, easy storage, and direct delivery to the field. This chapter contributes a methodology for encapsulation of somatic embryos by optimization of sodium alginate concentration and exposure time in calcium chloride solution. In addition to that, germination medium condition including medium strength, sucrose concentration, light/dark incubation period, and storage conditions were also noticed for the efficient storability of synthetic seeds.

Keywords Leaf sheath · Somatic embryos · Calcium alginate matrix · Artificial seeds · Sucrose dehydration

Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
BA	6-Benzyladenine
$CaCl_2.2H_2O$	Calcium chloride
GA_3	Gibberellic acid
MS medium	Murashige and Skoog, 1962

A. Shajahan (✉) · V. M. Mehaboob · A. Aslam
Plant Molecular Biology Laboratory, Department of Botany, Jamal Mohamed College, Tiruchirappalli, India

C. S. Raju
Department of Botany, Vivekananda College, Madurai, India

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1 Introduction

1.1 Curcuma *spp.*

Curcuma spp. belong to the family Zingiberaceae and include 60 species (Tyagi et al. 2007). It encompasses the most significant spice crops of *Curcuma longa* (turmeric) and *Curcuma amada* (mango ginger). In pharmaceutical industry, the rhizome is used for its stomachic, carminative, aphrodisiac, antipyretic, laxative, germicidal, anthelminthic, antioxidant, antimicrobial, anti-inflammatory, antidepressant, and hepatoprotective properties and plantlet aggregation inhibition activities (Nayak et al. 2011; Policegoudra et al. 2011). Turmeric is also known as golden spice and is highly valued for its underground rhizome. It contains a characteristic yellow-orange coloring matter, and it is widely used as a natural coloring agent in food industry (Salvi et al. 2002). The rhizomes have major anticancer compounds like curcuminoids [curcumin (1,7-bis(4-hydoxy-3-methoxyfenil)-1,6-heptadiene-3,5-dione), demethoxy-curcumin, and bismethoxy-curcumin] (Cousins et al. 2007). Mango ginger is consumed by human via its rhizomes and has been used to make pickles that have rich source of starch and a mongo-like smell with the presence of car-3-ene and cis-ocimene compounds (Gholap and Bandyopadhyay 1984). Rhizome has curcumin-free portion, which was identified to lower liver cholesterol in animals (Srinivasan et al. 2008). It also contains a labdane-type diterpenoid (labda-8(17), 12-diene-15, and 16-dial), which exhibits activity against tuberculosis (Singh et al. 2010).

1.2 Methods of Propagation

Curcuma is a sterile triploid species. The farmers utilize its rhizomes for cultivation and put it on the market for consumption. The vegetative propagation is slow to multiply and highly susceptible to physiological and temperature stress during storage (Policegoudra and Aradhya 2007). On the other hand, the rhizomes are infected by the bacterial wilt (*Ralstonia solanacearum*), fusarium yellow (*Fusarium oxysporum*), and soft/root rot (*Pythium* sp.) diseases (Prasath et al. 2011). Also, the rhizome is invaded by nematodes such as *Exigua ornithogalli* and *Trichobaris trinodata*. The larvae of these insects are developed inside the rhizome, which causes heavy loss in yield (Zapata et al. 2003). It inflicts serious economic loss to small and marginal farmers who depend on this crop for their livelihood. Therefore, alternative means of propagation methods are required to meet the increasing demand for rhizomes. Tissue culture technology offers biotic/abiotic stress-tolerant propagules for large-scale cultivation within a short duration under aseptic conditions.

1.3 Pretreatment/Callus Induction

Role of 2,4-D is highly effective for initiation of callus induction and somatic embryogenesis (Guo and Zhang 2005; Anandan et al. 2012). Preincubation

treatment of explants with lower concentration of 2,4-D in combination with BA to trigger the morphological competency of the cell by reception of the signals promotes further development of somatic embryos (Franklin et al. 2006; Dam et al. 2010). Prolonged culture in high concentration of 2,4-D leads to rapid cell division leading to callus induction (Venkov et al. 2000). We observed that the leaf sheath explants were cultured on MS medium encompassing with the 2,4-D in combination with BA-induced callus in *C. amada* (Soundar Raju et al. 2013) and on short duration incubation (20 days) on 2,4-D alone promote somatic embryogenesis in *C. longa* (Soundar Raju et al. 2015).

1.4 Somatic Embryogenesis

Somatic embryogenesis is a process without intervening callus stage (direct somatic embryogenesis) or from callus (indirect somatic embryogenesis) (Shajahan et al. 2016). A somatic embryogenesis method would also desirably provide for the ready introduction of plants into the field. 2,4-D has been found to serve as a trigger for inducing competent embryogenic cells. The reduction or removal of 2,4-D from the somatic embryo induction medium is essential for propagation of preembryogenic cells to the advanced stage of somatic embryos (Marsolais and Kasha 1985; Liang et al. 1987; Borkind et al. 1988; Ball et al. 1993). We found that the preincubated explants or calli were cultured in MS liquid medium in the presence of BA differentiated into somatic embryos (Soundar Raju et al. 2013, 2015). The plants derived from direct somatic embryogenesis usually are unicellular in origin and as a result genetically uniform. This system offers a number of advantages in crop improvement, as cost-effective and large-scale clonal propagation is possible using bioreactors, ultimately leading to automation of somatic seed production and development of artificial seeds (Soundar Raju et al. 2014, 2015) (Fig. 1).

1.5 Synthetic Seeds Technology

Synthetic or artificial seeds (or synseeds) have been described as "artificially encapsulated somatic embryos, shoots or other tissues which can be used for sowing under in vitro or ex vitro conditions" (Aitken-Christie et al. 1995; Germanà et al. 2011). Synthetic seed production technology that uses alginate encapsulation is considered an efficient choice for both propagation and germplasm storage of elite genotypes (Ara et al. 1999). The alginate coat shields the encapsulated explants from physical and environmental injury, reduces dehydration, and provides mechanical pressure to physically support the explants inside the gel matrix during storage (Ara et al. 2000). In addition, artificial seeds are a low-cost propagation method (Ghosh and Sen 1994; Saiprasad and Polisetty 2003). The success of synthetic seed technology depends on the quality of propagules. Somatic embryos represent a uniform developmental stage with reversible arrested growth and show high rates of conversion upon planting (Pinto et al.

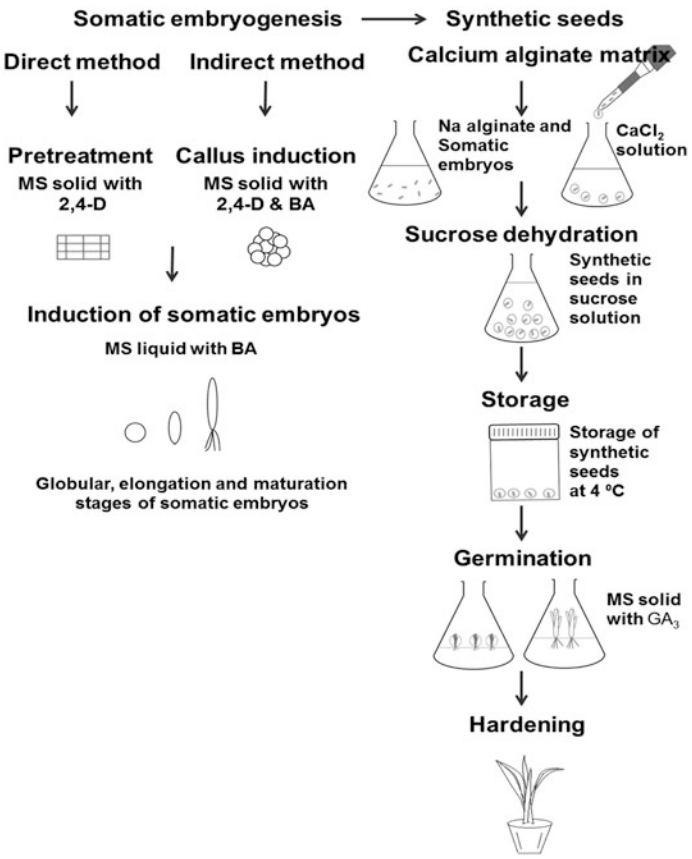

Fig. 1 Schematic diagram represents the induction of somatic embryogenesis and synthetic seed technology of *Curcuma* spp.

2008; Cheruvathur et al. 2013). However, the development of an efficient somatic embryogenesis system is a major prerequisite for the successful production of synthetic seeds (Bapat and Mhatre 2005). Once a reliable somatic embryo induction system is developed, the entire artificial seed production process can be automated (Ibaraki and Kurata 2001). However, the most desirable characteristic of the encapsulated explants is their ability to regrowth and form normal plants (Micheli et al. 2007; Parveen and Shahzad 2014). It is important to optimize methods for the storage, germplasm exchange, and regrowth of encapsulated explants (Sundararaj et al. 2010).

1.6 Calcium Alginate Matrix

Sodium alginate plays a key role in the structure of artificial endosperm that saves from destruction of the propagule. It has been extensively utilized in synthetic seeds

because of its low cost, gelling properties, low toxicity, and adequate firmness to usage (Bapat and Mhatre 2005). The texture of beads is robustly influenced by the gel matrix (sodium alginate) exposed to complexing agent (calcium chloride) which presumably helped an ion exchange between the Na^+ and Ca^{2+} (Singh et al. 2009). The optimum concentration of sodium alginate and exposure times to calcium chloride make on the preeminence synthetic seeds (Soundar Raju et al. 2016). Lower concentrations of sodium alginate result in the formation of soft and flimsy beads that are tricky to handle, while higher concentrations make isodiametric beads that are extremely hard, which considerably belated germination. High-level accumulation of calcium chloride in matrix might be causing toxicity on propagules (Nagesh et al. 2009; Gantait et al. 2012).

1.7 Sucrose Dehydration

Sugar accumulation in plant tissues is a renowned approach for dehydration stress; it stabilizes proteins and membrane bilayers (Zhu et al. 2006). Sustaining the viability of synthetic seeds after a long period of storage is an important prerequisite in the synthetic seed industry (Naik and Chand 2006; Micheli et al. 2007). After storage, the sucrose-dehydrated seeds showed a much higher germination rate than those of fresh seeds (Sundararaj et al. 2010; Cordeiro et al. 2014). This was proved in synthetic seeds of *C. amada* in our laboratory (Soundar Raju et al. 2016). The synthetic seeds dehydrated with lower concentration of sucrose (8.55%) provided sufficient sucrose to sustain the germination viability and development. No germination was observed when the synthetic seeds are dehydrated in high concentration of sucrose (17.11%). The synthetic seeds were stored at 4 °C for 120 days showed better germination rate. When the seeds were dehydrated with high sucrose level, negative response of germination was observed due to nonviability.

1.8 Tissue Culture Medium

Nutrient concentration plays an important role in growth and development of synthetic seeds (Gantait and Sinniah 2013; Mohanty and Das 2013). The medium of 1/2 strength had an impact in enhancing germination potential of synthetic seeds. MS medium at 1/4 strength might not be suitable, higher than 1/2 strength, 3/4 strength, and full strength beyond which synthetic seeds lost their viability. The germination percentage that was lower at other media strengths might be either detrimental due to overdose (nutrient toxicity) or inadequate due to low nutrient element content (Gantait et al. 2012). Sucrose displayed energy to the growing tissue; it has a pronounced effect on germination at 3%; absence of it or even reduction in concentration restricted the regrowth. Higher level of sucrose in medium-poor germination of synthetic seeds gradually turned white and became

nonviable. Germination and growth of somatic embryos into plants can be stimulated by the application of GA_3 in the culture medium (You et al. 2012; Manrique-Trujillo et al. 2013). Our study reports are evident on medium strength, sucrose concentration, and GA_3 for the germination of dormant somatic embryos in *Curcuma* spp. (Soundar Raju et al. 2014, 2015, 2016).

2 Requirements

2.1 Aseptic Establishment

1. In vivo-grown sprouts.
2. Tween-20.
3. Mercuric chloride ($HgCl_2$).
4. Sterilized distilled water.
5. Murashige and Skoog's (MS; 1962) medium.
6. Sucrose.
7. Benzyladenine (BA).
8. Indole-3-butyric acid (IBA).
9. Agar.

2.2 Somatic Embryogenesis

1. Leaf sheath explants (~1.5 cm long).
2. MS medium.
3. Sucrose.
4. 2,4-Dichlorophenoxyacetic acid (2,4-D)
5. Benzyladenine (BA).
6. Orbital shaker.

2.3 Synthetic Seeds Production

1. Mature somatic embryos (~0.5 cm length).
2. Sodium alginate solution.
3. Calcium chloride ($CaCl_2.2H_2O$) solution.
4. MS medium (with or without agar).
5. Sucrose.
6. 1 mL micropipette
7. Gibberellic acid (GA_3).

3 In Vitro Techniques

3.1 Aseptic Establishment

1. Sterilize the sprouts of 20-day-old *Curcuma* spp. in 2.0% (v/v) Tween-20 for 5 min and 0.1% (w/v) $HgCl_2$.
2. Wash the explants five times with sterilized distilled water.
3. Sprouts are culture on 0.8% (w/v) agar-solidified MS medium contain 3.0% (w/v) sucrose, supplemented with 1.0 mg L^{-1} BA and 0.5 mg L^{-1} IBA.
4. The cultures maintain at a temperature of 25 ± 2 °C, 16 h photoperiod (40 μm light intensity) provide by white fluorescent tubes and a relative humidity at 55–65%.

3.2 Somatic Embryogenesis

3.2.1 Pretreatment/Callus Induction

1. Collect leaf sheath explants from 3-month-old in vitro-raised plantlets.
2. Culture on MS solid medium supplemented with either 2.0 mg L^{-1} 2,4-D alone or 0.5 mg L^{-1} BA combination.
3. Maintain at above said conditions.

3.2.2 Direct Somatic Embryogenesis

1. Pretreated leaf sheath explants become swollen and soft.
2. Culture in/on MS medium either liquid or solid state.
3. Liquid culture maintain at 110 rpm under orbital Shaker with 25 ± 2 °C.

3.2.3 Indirect Somatic Embryogenesis

1. Collect soft, friable embryogenic callus.
2. Transfer to 250 ml Erlenmeyer flask contain 50 ml of 1/2 strength MS liquid medium with 3.0% sucrose and 0.3 mg L^{-1} BA.
3. Keep on Orbital Shaker at a rotation of 110 rpm.
4. Every 10 days, change the fresh medium containing flask.

3.3 Synthetic Seeds Production

3.3.1 Calcium Alginate Matrix

1. Select mature somatic embryos (~0.5 cm in length).

2. Transfer the 3% (w/v) sodium alginate solution prepared by 1/2 MS liquid medium.
3. Pipette the sodium alginate solution with somatic embryos using a 1 mL micropipette (tip diameter, 0.5 cm).
4. Drop the solution with a single embryo in sterile 100 mM $CaCl_2 \cdot 2H_2O$ solution.
5. Expose the calcium alginate beads to the solution for 15 min, with occasional gentle agitation for successful encapsulation of the matrix.
6. Wash the calcium-alginate beads in sterile distilled water and blot on filter paper to remove traces of $CaCl_2 \cdot 2H_2O$.

3.3.2 Sucrose Dehydration

1. Prepare the 8.55% (w/v) sucrose solution.
2. Take uniform-sized beads in sucrose solution on Erlenmeyer flask.
3. Keep on 24 h continuous shaking condition at 90 rpm.
4. Blot the beads on filter paper to absorb moisture content.

3.3.3 Synthetic Seed Storage

1. Take sucrose-dehydrated synthetic seeds.
2. Transfer the 15 seeds per sample container (50 mL).
3. Store at 4 °C.

3.3.4 Germination

1. After storage, remove the moisture content of synthetic seeds using filter paper.
2. Culture on 0.25 $mg\,L^{-1}$ GA_3-augmented 1/2 strength MS solid medium supplemented with 1.5% sucrose.
3. Incubate on 24 h darkness.

3.4 Hardening

1. Three weeks after germination, the plantlets transfer to 16 h light/8 h dark conditions.
2. Plantlets with 3–4 green leaves and 4–5 roots harden to pot mixture of autoclaved sand, soil, and vermiculate (1:2:1).
3. The plantlets maintain at 25 ± 2 °C under a 16-h light/8-h dark photoperiod.
4. After induction of new leaves, acclimatize to ex vitro conditions.

Fig. 2 Establishment of turmeric plants under in vitro conditions from in vivo-grown sprouts. (**a**) Initiation of sprouts on soil mixture. (**b**) In vivo-grown 20-day-old adventitious buds. (**c**) Multiple shoot formation on MS solid medium supplemented with 2.0 mg L^{-1} BA and 0.5 mg L^{-1} IBA under aseptic condition

4 Technical Reports

1. In *Curcuma* spp. leaf sheath excised from in vitro grown plants are used as a source of explants (Fig. 2a–c).
2. Culture leaf sheath explant on MS medium containing 2.0 mg L^{-1} 2,4-D for the formation of callus. Duration of explants above 20 days on the medium is required for induction of embryogenic callus. The medium without 2,4-D or short duration (5–20 days) culture will not show callus (Fig. 3a–c).
3. Subculture explants on solid medium containing 2.0 mg L^{-1} 2,4-D with a duration of 15–20 days. Preincubation causes direct somatic embryogenesis when culture on BA liquid medium (Fig. 3e–h).
4. Transfer preincubated leaf sheath explants/callus to MS liquid medium supplemented with 0.3 mg L^{-1} BA for the induction of maximum number of somatic embryos (direct and indirect). The induction of somatic embryos per explants will be significantly reduced if the concentration of BA is low or high.
5. Continued culture in MS liquid medium supplemented with 0.3 mg L^{-1} BA promotes the maturation of somatic embryos.
6. Although, BA at higher concentrations promote secondary somatic embryos.
7. Bipolar matured somatic embryos should be selected for synthetic seed technology; it shows uniform developmental stages and highest rate of conversion (Fig. 4a–d).
8. The size and shape of the beads are uniform in the 3% sodium alginate. The seeds produced by using 4% sodium alginate will form hardest coat. At lower concentrations of sodium alginate (1 and 2%), the beads are nonuniform and fragile, probably due high moisture content.
9. The size and shape of the beads are uniform when the 3% sodium alginate solution is exposed to 100 mM $CaCl_2 \cdot 2H_2O$ solution for 15 min. The shorter exposure time (5–10 min) will form nonuniform, fragile beads, and longer

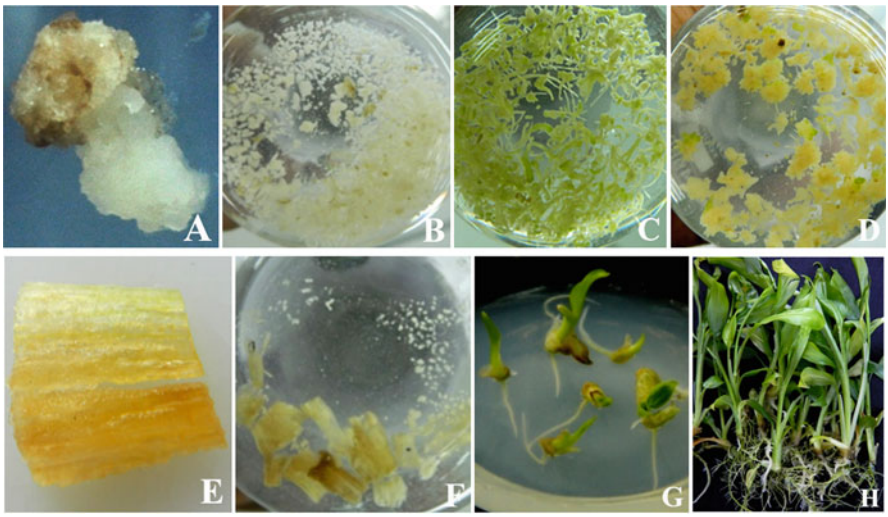

Fig. 3 Somatic embryogenesis of *Curcuma* spp. (**a–d**) Indirect somatic embryogenesis of *C. amada*. (**a**) Soft, friable nature of embryogenic callus, (**b**) establishment of embryogenic cell suspension culture, (**c**) development of bipolar somatic embryos and (**d**) formation of secondary somatic embryogenesis. (**e–h**) Direct somatic embryogenesis of *C. longa*. (**e**) Preincubated leaf sheath explants, (**b**) induction of embryogenic cell suspension from preincubated leaf sheath explants, (**c**) germinated somatic embryos, and (**d**) well-developed somatic embryo-derived plantlets

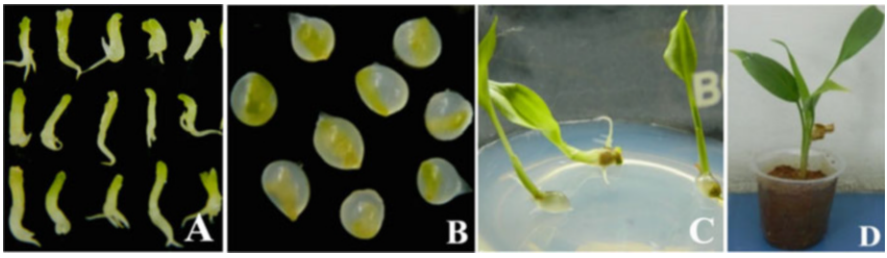

Fig. 4 Production of synthetic seeds from *C. amada*. (**a**) Matured bipolar somatic embryos. (**b**) Calcium alginate-coated somatic embryos. (**c**) Germinated synthetic seeds. (**d**) Ex vitro-acclimatized plants

exposure times (20–30 min) will produce hard beads with high accumulation of $CaCl_2$, which results in poor germination rate.

10. 1/2 strength MS medium is best for high rate of germination. Lower strength (1/4 MS) cause insufficient nutrition for germinated synthetic seeds and higher strengths cause the lower germination.
11. 3% sucrose in 1/2 MS medium enhances the rate of germination. The germination percentages are lower at other concentrations of sucrose. No artificial seed germination will occur on high sucrose (7%) concentration.

12. The maximum germination can be achieved on medium containing lower concentration of GA_3 (0.25 mg L^{-1}). Germination will be inhibited at higher GA_3 concentrations.
13. Transfer the well-developed plantlets (3–4 leaves and 4–5 roots) to potting mixture containing autoclaved sand, soil, and vermiculate (1:2:1) and maintain at 25 ± 2 °C under a 16-h light/8-h dark photoperiod for the acclimatization and high survival rate.
14. The results of in vitro propagation via somatic embryogenesis and encapsulation in calcium alginate matrix can be used as an efficient method for the mass propagation and germplasm conservation of *Curcuma* spp.

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Synthetic Seeds of Wild Beet: Basic Concepts and Related Methodologies

Saber Delpasand Khabbazi, Canan Yüksel Özmen, and Ali Ergül

Abstract Synthetic seeds are artificially encapsulated propagules that mimic true seeds in agriculture. Although a variety of plant materials, such as shoot tips, axillary buds, callus, micro cuttings, and protocorm-like bodies, are used in the production of synthetic seeds, somatic embryos are the most widely used explants in the production of these seeds. Synthetic seeds compete with traditional approaches to preserve the germplasm of threatened plant species. The resulting progenies are the true clones of the main plant, thus preserving the intactness of the genetic background. Due to poor germination and low seed amount, wild *Beta* species are exposed to the risk of extinction. Wild relatives of *Beta* have agronomically important properties such as resistance to diseases and abiotic stresses. Numerous attempts have been made to give these traits to sugar beet crop through conventional breeding methods. Despite the importance of synthetic seed for wild beets, it has not yet been investigated. The production of synthetic seeds ensures the conservation and availability of wild germplasm of the genus *Beta* for cytogenetic and breeding studies.

Keywords Artificial seeds · Biotic and abiotic stress · Genus *Beta* · Germplasm conservation · Resistance genes

1 Introduction

Seeds are zygotic embryonic plants produced after fertilization in flowering plants. Seeds connect different generations of plants and ensure the maintenance and transfer of plant genetic material in nature. These structures are enclosed with protective layers to keep the embryo safe during storage and dispersal (Bewley and Black 1985).

Seeds are essentially composed of an embryo and protective layers. True seeds contain endosperm tissues that provide the nutrients necessary for germination. Endosperm stores various substances mainly including starch, proteins, and oils.

S. D. Khabbazi · C. Yüksel Özmen · A. Ergül (✉)
Biotechnology Institute, Ankara University, Ankara, Turkey
e-mail: ergul@ankara.edu.tr

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However, in some species, cotyledonary leaves are the source of these components. Seeds are the basis of agriculture and, when the necessary factors for germination are provided, they produce a plant similar to the main plant.

Depending on plant species and conditions, the production of true seeds can be time-consuming, arduous, impossible, or costly. Synthetic seeds have great potential as an alternative to true seeds, especially due to low production costs and long-term storage (Roy and Mandal 2008). These seeds can be handled, stored, transported, and planted like true seeds (Sharma et al. 2013).

The term "synthetic seed" was first defined as an encapsulated single somatic embryo by Murashige (1977). In the early studies, the concept was limited to plants where somatic embryogenesis could be demonstrated; however, it was later on extended to any in vitro-derived propagule due to the recalcitrance of some species to somatic embryogenesis (Bapat et al. 1987). Synthetic seeds are also known as artificial seeds, synseeds, and manufactured seeds. Somatic embryos are often used as propagule in the production of synthetic seeds; however, cell aggregates, shoot buds, auxiliary buds, or any other structure capable of giving rise to a plant can also be used. Furthermore, these seeds can hold this ability for a long time and can be stored (Ara et al. 2000; Saiprasad 2001; Daud et al. 2008).

Synthetic seed technology is a suitable alternative method for proliferation of commercially important plants. These tissue culture-originated artificial seeds are economically preferred for the propagation of hybrids generated through breeding approaches (Rihan et al. 2017). Synthetic seeds, however, can be used to proliferate the species that hardly reproduce through generative propagation and are at risk of extinction.

The wild relatives of sugar beet (*Beta vulgaris* spp. *vulgaris*) are invaluable germplasm reservoirs that are in danger of extinction. Although synthetic seeds have been produced for more than 30 years, no attempt has been made to utilize synthetic seed technology for wild beet species. However, in situ and ex situ efforts have been made to protect these invaluable genotypes (Frese et al. 2001).

In this chapter, we emphasize the importance of wild beet relatives in the genus *Beta* and review the synthetic seed technology to evaluate the use of this approach for wild beet accessions.

2 Origin and Development of Synthetic Seeds

When Steward et al. (1958) and Reinert (1958), at the same time, reported the first somatic embryogenesis in the carrot plant (*Daucus carota*), most probably the idea of synthetic seed production created. However, the first report of plant propagation by somatic embryos was presented by Murashige (1977). He succeeded to obtain the surviving plants originating from somatic embryos in vitro conditions. Studies continued to accelerate the process of developing plants from somatic embryos for commercial applications. Drew (1979) delivered the carrot somatic embryos in a liquid drilling system; however, the results were disappointing as he developed only three carrot plants in carbohydrate-free media. The first synthetic seed production goes back to a report by Kitto and Janick (1982). They encapsulated multiple

somatic embryos of carrot in a polyoxyethylene glycol and desiccated the embryos. Polyoxyethylene is a readily soluble chemical in water and after drying forms a thin layer. In addition, it is not toxic to the embryo and does not allow growth of the microorganism and, therefore, later was also used to encapsulate the celery (*Apium graveolens*) embryos (Kitto and Janick, 1985). A polyethylene glycol (PEG)-based mixture was also applied to coat the carrot somatic embryos and embryonic calli. Following the dehydration procedure, the survival rate was scored by placing the coated embryos on culture medium allowing them to rehydrate (Janick et al. 1993). Since the first studies, many efforts have been made to produce synthetic seed in various plant species (Ravi and Anand 2012). Survival percentage of encapsulated embryos was enhanced by modifications applied in the encapsulation matrix and speed of dehydration. Various propagules have been utilized to establish the system for the species recalcitrant to somatic embryogenesis.

3 Types of Synthetic Seeds

The somatic embryos used for the production of synthetic seeds may or may not be encapsulated. The state of quiescence in uncoated somatic embryos defines the usage of these seeds. If the uncoated embryo is non-quiescent, it is used for the in vitro micropropagation of plant, and if quiescent, it is used for the germplasm storage. The encapsulated somatic embryos are divided into two types of hydrated and dried seeds. The encapsulated somatic embryos may be non-quiescent or quiescent if the seed coat is hydrated or desiccated, respectively. The quiescent somatic embryos coated with dehydrated artificial coatings are the most resembling synthetic seeds to the conventional ones in terms of handling and storage.

The vigor of the seedlings obtained from the desiccated embryos is higher than that obtained from the hydrated embryos. It is ideal if the synthetic seed is produced through encapsulated desiccated embryos (Pond and Cameron 2003). Prior to encapsulation process, somatic embryos are hardened to tolerate the desiccation which induces the quiescence. However, desiccated seeds are only produced if the somatic embryos are tolerant to dehydration. In some species, the generated somatic embryos are sensitive to water deficiency; thus, the embryo ought to be coated with hydrogels (Magray et al. 2017). Desiccation degree is determinative and depends on the developmental stage of the embryo. If the embryo is mature, the drying process should be carried out rapidly, and if it is immature, it is opted to implement the process slowly (Senaratna et al. 1990).

4 Wild Beets: Synthetic Seed and Applications

4.1 Species of the Genus Beta

Based on the crossing ability between cultivated beet and other genotypes, the genus *Beta* divides into three gene pools: the primary gene pool contains the cultivated beet

and other species which can easily cross; the secondary gene pool includes the species that despite the crossability with cultivated beet generate sterile progenies; and the tertiary gene pool members generate hybrids only by human intervention and artificial crossing (Jassem 1992; Kadereit et al. 2006; Frese 2010) (Table 1). However, an earlier classification had placed *B. nana* in a separate section, thus dividing the genus *Beta* into four sections as *Beta*, *Corollinae*, *Nanae*, and *Procumbentes* (Ford-Lloyd 2005). The Eastern Anatolia-Western Caucasus crossing region is accepted as the origin and resource of genetic diversity for *Beta* and *Corollinae* (Boughey 1981). Section *Nanae* is distributed in the mountainous regions of Greece, whereas the members of the section *Procumbentes* spread far away in the Canary Islands, coastal areas of North-West Africa, and Southeastern Spain (Frese 2010).

4.2 Employment of the Wild Beet Genotypes

The interest in wild beet genotypes as the genetic resources for improving the sugar beet germplasm has been increasing since the late 1800s, and the values of these genotypes have been well-demonstrated (De Bock 1986; Doney and Whitney 1990; Van Geyt et al. 1990; Lewellen and Skoyen 1991; Doney 1993). Since that time, breeders have been employing these germplasms to improve the agronomic traits of the sugar beet crop. There are committees established to study and protect the germplasm of the genus *Beta*. Very comprehensive information on the genotypes of *Beta* is available at the Genetic Resources Information Network (GRIN) Database of National Plant Germplasm System (NPGS). More than 22% of the available *Beta* accessions are the *B. maritima* accessions which are well-characterized and most useful accessions in the breeding of sugar beet (Biancardi et al. 2010).

4.3 Wild Beets Are the Reservoir for Resistance Genes

Sugar beet yield is affected by different biotic and abiotic factors. The cyst nematode (*Heterodera schachtii* Schm.), leaf spot disease (*Cercospora beticola* Sacc.), and *Beet necrotic yellow vein virus* (BNYVV) are among the most destructive pests and disease agents of sugar beet crop that reduce root yield and sugar content (Weiland

Table 1 Gene pools of the genus *Beta* (Jassem 1992; Kadereit et al. 2006; Frese 2010)

Gene pool 1	Gene pool 2	Gene pool 3
Beta vulgaris ssp. *vulgaris*	*Beta corolliflora*	*Beta patellaris*
Beta vulgaris ssp. *adanensis*	*Beta macrorhiza*	*(Patellifolia patellaris)*
Beta vulgaris ssp. *maritima*	*Beta lomatogona*	*Beta procumbens*
Beta macrocarpa	*Beta trigyna*	*(Patellifolia procumbens)*
Beta patula	*Beta intermedia*	*Beta webbiana*
	Beta nana	*(Patellifolia webbiana)*

and Koch 2004; McGrann et al. 2009; Biancardi et al. 2010). Sugar beet cyst nematode is spread over more than 40 countries and causes major product losses (McCarter 2008). However, some *Beta* species carry cyst nematode resistance genes that can be employed in breeding programs. *C. beticola* is a fungal pathogen known as the most damaging sugar beet disease worldwide, corresponding to leaf spot disease in sugar beet (Weiland and Koch 2004). Depending on the severity of the disease, *C. beticola* reduces the sugar yield by 1–55% (Rossi et al. 1995; Altınok 2012). The soil-borne disease of rhizomania is caused by BNYVV, which is transmitted by *Polymyxa betae* Keskin (Keskin 1964). Rhizomania causes yield losses of up to 80% in the sugar beet crop and the only way to control the disease is to cultivate resistant varieties (Tamada and Baba 1973).

Wild relatives of genus *Beta* contain agronomically important characteristics and can be exploited in introgression of desirable traits into cultivated beet crop (Table 2). Being a member of the section *Beta*, *B. v.* ssp. *maritima* is a very close and cross-compatible relative of *B. v.* ssp. *vulgaris* (Biancardi et al. 2012). It owns valuable traits such as resistance to *H. schachtii* and is the widely deployed wild-type genotype in sugar beet breeding studies (Panella and Lewellen 2007). In addition, *B. maritima* is a source of resistance to *Cercospora leaf spot* disease, so it has been used to develop resistant varieties (Munerati et al. 1913). Although resistance to *Cercospora* has been reported in all of the *Corollinae*, *Procumbentes*, and *Beta* sections, the resistance degree is highly different between them. Among species, *B. maritima* has the most pronounced degree of resistance, so it is widely used in sugar beet breeding approaches (Munerati 1932; Panella and Frese 2000; Skaracis and Biancardi 2000; Luterbacher et al. 2004). In contrast to *B. maritima*, *B. nanae* and *B. macrorhiza* accessions are susceptible to *Cercospora* (Coons 1954). *B. maritima* also could be a reliable resource for resistance genes of rhizomania, and among these genes, *Rz1* and *Rz2* are generally used in the commercial development of resistant varieties (Scholten et al. 1999). Wild beet genotypes are also invaluable resources of tolerance to abiotic stresses such as drought, salt, and frost which could be exploited to improve the quality of growth in sugar beet under adverse climatic conditions (Frese et al. 2001). The *Corollinae* section, in particular *B. corolliflora*, is thought to be an important germplasm against abiotic stresses. However, the low homology percentage of chromosomes between different gene pools may be an obstacle in the transmission of the desired genes (Jung and Wricke 1987; Van Geyt et al. 1990).

4.4 The Contribution of Synthetic Seeds to Breeding Programs

The first production of the sugar beet crop was restricted to Northern Europe, a temperate climate and a relatively disease-free zone; therefore, small selection pressure was applied against the pathogens. Following the cultivation in other

Table 2 Transition of resistance genes from wild beet genotypes to sugar beet crop via conventional breeding approaches

	Resource of resistance	*B. vulgaris* hybrid accessions obtained through interspecific breeding	Biotic factor	References
Beta section	*B. maritima*—WB42, Rızor or Holly, WB41, WB258, R36, R22 (PI 590791)	*B. vulgaris*—Holly-1–4, R104	BNYVV	Lewellen and Whitney (1993), Pelsy and Merdinoglu (1996), Scholten et al. (1999), Gidner et al. (2005), Grimmer et al. (2008a, b)
	B. maritima	*B. vulgaris*—CN921-515 (Reg. No. GP-295, PI 669447) and CN921-516 (Reg. No. GP-296, PI 669448)	Cyst nematode	Richardson (2018)
	B. maritima—WB242 (PI 546413), N499 (PI 599349)	*B. vulgaris*—CN12 (PI 636338), CP07 (PI 632288), CP08 (PI 6322889)	Cyst nematode	Heijbroek et al. (1977), Lewellen (2004, 2006)
	B. maritima	*B. vulgaris*—PI 357354, PI 518303, PI 546413, PI 504180, and PI 546413	Cyst nematode	Panella and Lewellen (2007)
	B. maritima	*B. vulgaris—M66, WB258 (PI 546426)*	Root-Knot Nematode	Yu (1997, 2002)
	B. maritima	*B. vulgaris*—Rovigo (R148, R581, etc.), varieties "Cesena" and "Mezzano"	*Cercospora*	Munerati (1932), Biancardi and De Biaggi (1979)
	B. maritima—WB97, WB242	*B. vulgaris*—CP01 and CP02	Powdery mildew	Lewellen (2000)
	B. maritima—WB178, PI 546403	*B. vulgaris*—83 W304	Yellow wilt	McFarlane (1984)
	B. maritima—PI 546409 (WB185), PI 540625 (WB879)	*B. vulgaris* (SP6822 X WB879)	*Aphanomyces*	Yu (2004)

(continued)

Table 2 (continued)

	Resource of resistance	*B. vulgaris* hybrid accessions obtained through interspecific breeding	Biotic factor	References
Procumbentes section	*B. procumbens*	*B. vulgaris* (B883, ANl-65-2, AN101)	Cyst nematode	Van Geyt et al. (1988), Lange et al. (1988, 1993), Salentijn et al. (1992)
	B. procumbens—AU6-1-4 and D3-2-13; *B. patellaris* – B1-1-54	*B. vulgaris*—Holly-1–4	*Cercospora*	Mesbah et al. (1997)
	B. patellaris—A5-1-7 and B1-1-192	*B. vulgaris*—Holly-1–4	BNYVV	Mesbah et al. (1997)

regions with different climatic conditions, various diseases emerged and affected crop yield (Lewellen 1992). Although numerous genetic improvements have been made to date, susceptibility to disease still threatens crop yields and production worldwide (Table 2). Wild beet accessions have great potential to expand the germplasm pool of sugar beet. Wild *Beta* species are evaluated in terms of important agronomic characteristics to be used in sugar beet breeding. These accessions indicate different degrees of resistance to biotic and abiotic factors. Initial efforts to screen wild beet genotypes for pathogen resistance were made in the early 1900s. The first documented report of using the *B. vulgaris* ssp. *maritima* in breeding studies of cultivated beet belongs to Munerati et al. (1913) who found *B. maritima* as a source of resistance to *Cercospora leaf spot* disease (Biancardi et al. 2010). However, various resistance genes were later on transferred to *B. vulgaris* through interspecific hybridization. The obtained interspecific hybrids were resistant to different biotic factors (Table 2). The synthetic seed technology contributes to the breeding of cultivated accessions. The preparation of synthetic seeds for *Beta* wild relatives ensures the presence of these accessions for breeding programs.

Sugar beet is a biennial crop and has to be exposed to the prolonged cold of winter for flowering and seed production (Letschert 1993). However, wild-type relatives are naturally annual, and production of the flowering stem is triggered in the first year (Biancardi et al. 2010). Depending on the geographical origin and altitude, a perennial growth pattern is also possible in wild beet genotypes (Marlander et al. 2011). In vitro production of synthetic seeds allows researchers to shorten the breeding process by applying equivalent but controlled conditions to stimulate vernalization.

Simultaneous flowering time is very important for successful plant-pollinator interactions (Elliott and Weston 1993; Alcaraz et al. 1998). The lack of synchronicity in flowering, especially in different genotypes, makes the hybridization of wild

and cultivated species difficult (Alibert et al. 2003; Cuguen et al. 2005). Application of synthetic seeds and human intervention to adjust the cultivation time enhance the chance of fertilization and seed production. The mass production of clonal plants with homogeneity in the genetic background causes simultaneous flowering in the population. In addition to homogeneity in flowering, the production of large amounts of pollen is another feature that breeders will appreciate when they intend to convey the desired characteristics between species. Compared to the monogerm seeds, the multigerm *Beta* accessions produce more pollen (Alibert et al. 2003; Biancardi et al. 2010). Therefore, the selection of superior genotypes that match the breeding objectives is an important step before hybridization.

The production of inbred lines is necessary for the development of hybrid seeds. However, inbreeding is sometimes not possible due to genetic barriers and the presence of allogamy in many species. Sugar beet is primarily self-incompatible, and the self-pollination is rare among wild beets. This feature was used by breeders to increase and maintain heterosis in multigerm varieties prior to the discovery of cytoplasmic male sterility (Owen 1942). Large-scale conservation and micropropagation of selective rare hybrid genotypes have attracted interest in the production of synthetic seeds. The production of synthetic seeds can eliminate the genetic segregation because of the participation of only one parent, as well as the use of somatic cells in seed production. These seeds are the actual clones of the sampled plant, so it can also be considered in the maintenance of the unstable sterile genotypes, genotypes that have difficulty in germination and transgenic plants (Gantait et al. 2015).

Hybridization of species distributed in different gene pools is limited due to existing genetic barriers (Abe and Tsuda 1987; Jung and Wricke 1987; Van Geyt et al. 1990). However, there are successful hybridization reports between the species of different sections (Table 2). The obtained interspecific hybrids might lack one or more chromosomes leading to aneuploidy. These individuals will be used in cytogenetic and biotechnological studies of *Beta* species (Savitsky 1960, 1975; Yu 1983, 2005; Heijbroek et al. 1988; Sandal et al. 1997). Thus, the protection of the desired genotypes between such hybrids requires an asexual multiplication technique. Artificial seed technology can protect and reproduce the desired progeny for future uses in breeding programs.

5 Germplasm Conservation of Wild Beet Species

True seeds are consisting of an embryo, nutritive tissues, and protective layers. The embryo and nutritive tissues of true seeds are covered with a seed coat which keeps the embryo quiescent and tolerant to adverse climatic conditions that have been the source of inspiration for the preservation of the germplasm in gene banks. Zygotic seeds are reliable sources to preserve germplasms in repositories. Furthermore, the incidence of pathogenic infestations and high metabolic activities affect the period of seed storage. Unlike the true seeds, synthetic seeds do not necessarily contain nutritive tissues or seed protective layers and the state of quiescence might be

different in these seeds. Therefore, depending on the purpose of use, the structural complexity of synthetic seeds is defined. Seed coats of synthetic seeds not only help to the keep the propagules from pathogenic diseases but also establish a safe seedbed in the soil. It protects the propagules from drought and other unfavorable conditions and also keeps the seed safe during the transportation and storage (Ara et al. 2000).

True seeds of the wild beet species are coated with a thick and excessively indented testa which causes poor germination (Coons 1975). The perennial pattern of growth and the low germination rate affect the multiplication of wild beet relatives in natural habitats (McGrath et al. 2007; Marlander et al. 2011). The difficulties in germination and proliferation, especially among the species of *Corollinae*, challenge the survival of wild beet species (Frese et al. 2001). To date, in situ and ex situ efforts have been made to save these genotypes in their natural distributed areas or botanical gardens (Ren et al. 2012). However, the need to accelerate and improve the propagation process and efficiency is still felt.

Unlike the true seeds which produce following the sexual reproduction and undergo the genetic recombination process, the intactness of the parental genetic background is protected in synthetic seeds. Propagules used in the production of synthetic seeds are generated in aseptic conditions; therefore, the resulting seeds are pathogen-free and thus are superior to the traditional methods in which diseases encounter severe threats to the stoked genotype. Moreover, the international exchange of plant material would be easier and faster across the country borders and successfully contribute to the control of plant diseases (Daud et al. 2008; Nyende et al. 2005). Recently the in vitro multiplication of wild beet genotypes was investigated to assess the in vitro regeneration potential of different species of the genus *Beta* (Ergül et al. 2018). Results indicated the multiplication capability of the selected genotypes when were subjected to cytokinin and gibberellin growth regulators. In an early study, the callus induction potential of the wild genotypes of *Beta* sections was evaluated (Yu 1989). Despite the conducted studies, the assessment of somatic embryogenesis in these species yet remains. Somatic embryos are frequently used as proper explants in the production of synthetic seeds; therefore, the importance of studies over the somatic embryogenesis in wild beet species is emphasized (Skaracis 2005).

Production of synthetic seeds will provide a sufficient quantity of wild beet accessions to ensure the germplasm conservation. This technique will ease the renewal of germplasm in gene banks, and the germplasm continuity will be ensured; moreover, the costs of germplasm maintenance in the gene banks will be reduced efficiently.

6 Synthetic Seed Production and Storage Methods

6.1 Explant Materials

A variety of propagules have been utilized in the production of synthetic seeds (Fig. 1). Propagules are divided into two categories of unipolar and bipolar. Both

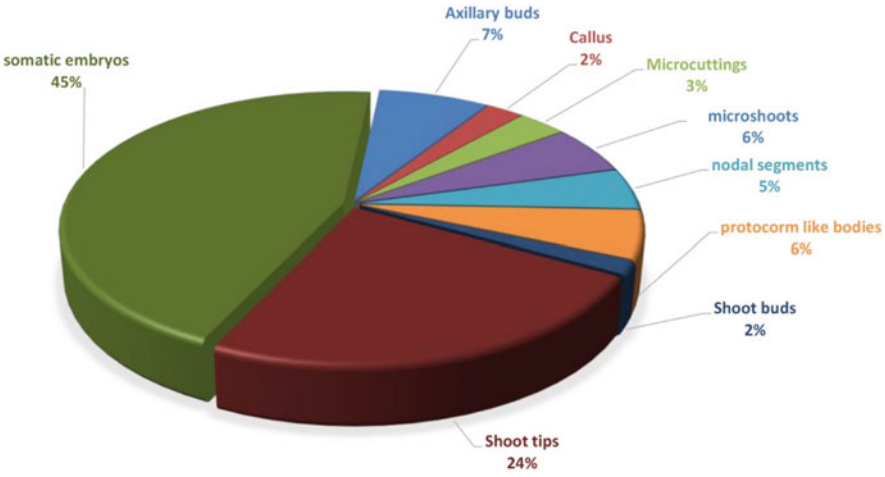

Fig. 1 The pie chart depicts the propagules used in synthetic seed production and their relative contribution to synthetic seed production studies (Reddy et al. 2012)

types are used in the production of synthetic seeds and have pros and cons. Bipolar propagules such as somatic embryos contain shoot and root apical meristems and generate the plant in a single stage (Ara et al. 2000). Such seeds can be used as hydrated or dried. Seeds carrying the somatic embryos have a strong capacity for reproduction and can maintain their regenerative potential for a long time leading to a uniform plant production (Leroy et al. 2000). The germination rate of the somatic embryos is different from that of zygotic embryos so that the zygotic embryos are superior to synthetic seeds in germination rate (Lulsdorf et al. 1993; Attree et al. 1994). Nevertheless, the encapsulation method and the plant genotype are determining factors in germination rate of the seeds (Cartes et al. 2009). The resulting plants originating from bipolar propagules may cause somaclonal variations. Moreover, asynchronous and late maturation features of embryonic poles are among the factors restricting the usage of somatic embryos in the production of synthetic seeds (Castellanos et al. 2004). On the other hand, unipolar propagules contain only a shoot or a root pole and are superior to the bipolar ones in terms of genetic fidelity and a vast range of vegetative explants. Unipolar explants can be any of the apical tips, axillary buds, micro shoots, micro bulbs, microtubers, corms, rhizomes, meristemoids, cell aggregates, and primordia biological materials with different levels of complexity and conversion ratios (Sharma et al. 2013).

Although propagules such as shoot apical meristems, axillary buds, and micro shoots do not contain root apical meristem, these are encapsulated to generate seeds. However, prior to the encapsulation process, explants are subjected to the root inducing chemicals. Some studies reported the possibility of root induction and conversion of the buds to plantlets when cultivated on white's rooting medium without chemical pretreatments (Bapat and Rao 1990; Ganapathi et al. 1992). Based on a study, the conversion of the encapsulated apical tips was more than

axillary buds; however, a later study indicated the conversion of 100% for axillary buds when encapsulated in a suitable matrix (Capuano et al. 1998; Lata et al. 2009).

Plant species respond differently to synthetic seed production that emphasizes the effect of genotype and plant species. The literature review shows that somatic embryos as the most corresponding propagule have been employed in the majority of the studies including vegetable crops, spices, and plantation crops, ornamental plants and orchids, medicinal plants, forage legumes, fruit crops, and forest trees (Reddy et al. 2012). Following somatic embryos, shoot tip explants are the second most widely used propagule; however, other explant sources have been less frequently used (Fig. 1). Both somatic embryos and shoot tip explants were used as plant material in the production of synthetic seeds for *B. vulgaris*, which can also be recommended for wild relatives of *Beta* (Saunders and Tsai 1999; Rizkalla et al. 2012).

6.2 Encapsulation Chemicals and Processes

Agents such as agar, agarose, alginate, polyox, polyco 2133, guar gum, tragacanth gum, gelrite, carrageenan, carboxymethyl cellulose, polyacrylamide, nitrocellulose, and sodium pectate ethyl cellulose have been tested for the encapsulation of synthetic seeds till date (Ara et al. 2000; Saiprasad 2001; Lambardi et al. 2006). Among these chemicals, sodium alginate has been found as the most suitable agent for encapsulation of somatic embryos due to its low cost, low toxicity, and quick gelling properties (Saiprasad 2001). This chemical was used to encapsulate the *B. vulgaris* somatic embryo and shoot tip propagules at concentrations of 2% and 4%, respectively (Saunders and Tsai 1999; Rizkalla et al. 2012). Sodium alginate can protect the biologic material for a longer time when compared to other agents such as agar. The firmness of the seed coat is determined by the ratio of sodium ions exchanged with calcium in $CaCl_2 \cdot H_2O$ solution (Daud et al. 2008). The literature review indicates that the most satisfying results are obtained once explants are encapsulated with 3% sodium alginate and 100 mM $CaCl_2 \cdot H_2O$. In the majority of these studies, the regeneration frequency was noted more than 90% (Singh and Chand 2010; Ahmad et al. 2012; Sakhanokho et al. 2013; Varshney and Anis 2014).

Structure of the synthetic seeds imitates that of true seeds. The biologic material in synthetic seeds represents the zygotic embryo in true seeds (Cartes et al. 2009). Synthetic endosperms are comprised of MS culture medium supplemented with growth regulators such as cytokinin and auxins, minerals and vitamins, gelling chemical, and anti-pathogenic components (Ravi and Anand 2012).

Synthetic seeds are single-layered, double-layered, or hollow bead structures. To produce single-layered seeds, the in vitro originated plant materials are mixed with a proper hydrogel. Alginate is the most frequently used coating agent employed in the concentration of 0.5–5%. After alginate dissolves in double distilled water or liquid nitrogen, the solution is utilized in the production of the beads containing propagules. The beads are then treated with a complexion agent such as calcium chloride ($CaCl_2 \cdot 2H_2O$). It is important to obtain round and firm calcium alginate beads.

The concentration of sodium and calcium together with the complexion time affect the permeability and rigidity of the beads and may be different between plant species. Generally, treatment of beads with 3% (w/v) sodium alginate and 100 mM calcium chloride for 20–30 min is reported as the most suitable combination for seed production (Sarkar and Naik 1998; Tabassum et al. 2010; Ahmad and Anis 2010; Ozudogru et al. 2011; Alatar and Faisal 2012; Hung and Trueman 2012a, b). Low alginate concentrations (<3%) interfere with solidification and high concentrations (5–6%) result in a very hard coating that delays germination (Larkin et al. 1998; Ahmad and Anis 2010; Sharma et al. 2009a, b).

The content of the matrix, such as nutrients and growth regulators, influences the success of germination and conversion of the encapsulated explant (Chand and Singh 2004; Sundararaj et al. 2010). Synthetic seeds are highly susceptible to microbial infections, so various antimicrobial agents are added to the gel matrix to reduce infection (Saiprasad 2001; Wang et al. 2007).

The addition of activated carbon to the matrix gel enhances explant conversion and vigor. Activated carbon adsorbs toxic products such as phenolic compounds that can damage encapsulated propagule; moreover, it helps the diffusion of nutrients and gases. It contributes to decomposition of alginate and enhanced respiration of the biological material, thereby prolongs the storage time. Additionally, the activated carbon retains the nutrients and releases them gradually which provides a long-term supply of essential nutrients for the propagule. Pretreatment of synthetic seeds with potassium nitrate makes an impressive contribution to the production of shoots and roots from coated propagules (Sharma et al. 2013).

Double layering the plant material is proposed to increase the protection of encapsulated propagules. Once the single layer seeds are produced, they can be coated with the same concentration of sodium alginate and then coated with the treatment of $CaCl_2 \cdot 2H_2O$. Double-layered synthetic seeds have the same properties of the single-layered ones; however, double encapsulation provides better protection (Micheli et al. 2002; Pinker and Abdel-Rahman 2005).

When the position of the propagule in the bead is compared between conventional synthetic seeds and true seeds, the adjacent position of the propagule to bead surface makes the protection fragile in synthetic seeds. Hollow beads are promising tools to assure the protection of encapsulated propagules; however, the process is laborious and costly, and the success of this method is still controversial (Winkelmann et al. 2004; Pourjavadi et al. 2006).

6.3 Storage of Synthetic Seeds

Storage of synthetic seeds is carried out for a variety of purposes, such as the germplasm transport between countries, proliferation, and protection of invaluable germplasms. Researchers investigate the ideal conditions for the storage of synthetic seeds. Storage temperature and matrix components are the most important factors affecting the conversion rate; however, the effect of species on the storage is also

decisive. Generally, 4 °C is the most suitable temperature for short-term storage of synthetic seeds including *B. vulgaris* (Saiprasad and Polisetty 2003; Kavyashree et al. 2006; Singh et al. 2007; Faisal and Anis 2007; Pintos et al. 2008; Sharma et al. 2009a, b; Ikhlaq et al. 2010; Tabassum et al. 2010). However, several studies on some tropical and subtropical crops have reported that higher temperature (25 °C) is required for bead storage (Srivastava et al. 2009; Sundararaj et al. 2010; Mishra et al. 2011). Although wild *Beta* species have not been investigated for synthetic seeds, there are reported studies over *B. vulgaris* spp. *vulgaris*. In vitro storage of sugar beet synthetic seeds was evaluated after addition of osmotic agents to the MS medium (Rizkalla et al. 2012). According to the results, the addition of 0.05 M mannitol or sorbitol to the medium increased seed survival during in vitro storage but disrupted growth quality. This result is consistent with Westcott (1981), which previously reported the toxic effects of mannitol. In an early study, the effect of cold storage of the beads was investigated, and the results showed that the cold treatment did not improve the conversion rate and also slowed down the rate of development (Saunders and Tsai 1999).

For long-term storage of synthetic seeds, dehydration and cryopreservation storage techniques are used. Cryopreservation of the propagules can only be useful if the formation of intracellular ice crystals is avoided; otherwise, harmful effects prevent cell survival. Various techniques of cryopreservation inclusion of simple desiccation, encapsulation-dehydration, the two-stage freezing, vitrification, and encapsulation-vitrification have been employed to date. Pretreatment of encapsulated biological tissues in a medium supplemented with high concentrations of sucrose results in progressive water withdrawal of the coated propagule. Additional dehydration of the encapsulated explants increases the concentration of sucrose results in a glass transition during cooling to ultralow temperatures (–196 °C) and subsequently fatal damages to the cells (Engelmann and Takagi 2000). Figure 2 summarizes the approaches used to store synthetic seeds.

7 Difficulties in the Production of Synthetic Seeds

Large-scale and cost-effective synthetic seed production requires investigations of high-quality propagules and encapsulation methods. Since the first synthetic seed production report, many improvements have been made. Despite the impressive advantages, this technique is currently facing limitations that challenge commercial production. Lack of dormancy in synthetic seeds causes limitations during the storage period. Especially because these seeds are stored at lower temperatures, the vitality and conversion rate reduces over time. Among the biological materials studied, somatic embryos are superior to others because of their potential in the production of the shoot and root system in one step. However, in addition to improper maturation of somatic embryos, synchronic deficits in the development of somatic embryos make the application of somatic embryos difficult (Reddy et al. 2012; Hung and Trueman 2012a, b). Somatic embryogenesis may be difficult due to the existing recalcitrance of

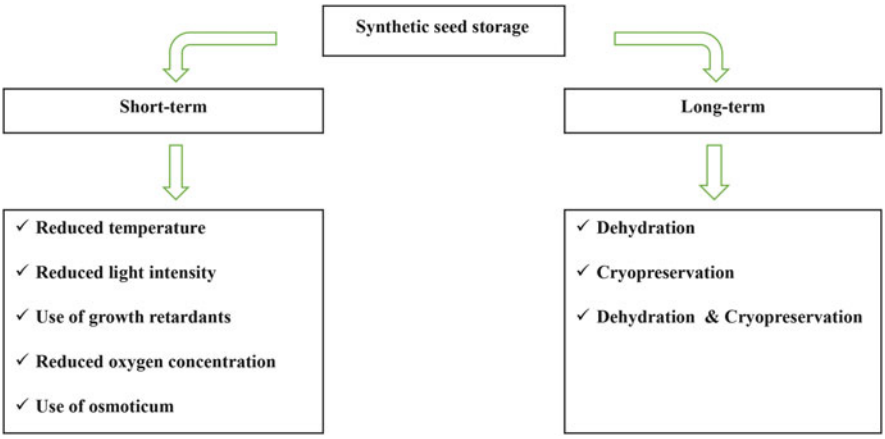

Fig. 2 Various techniques are used to store synthetic seeds depending on the purpose of storage

some plant species, thus encouraging the use of other explant sources in the production of synthetic seeds. Although such explants are promising tools, the creation of the root system is a complex challenge in this approach. In most woody plant species, single-step rooting is the major obstacle to non-embryogenic coated propagules (Chand and Singh 2004; Naik and Chand 2006; Hung and Trueman 2012a).

One of the main problems of synthetic seeds is the practicality of these seeds during sowing under ex vitro conditions. In addition to the oxygen supply and nutrient deficiency, the presence of various pathogens in commercial substrates such as soil or vermiculite causes infection risks and other limitations (Jung et al. 2004; Rihan et al. 2012). However, the successful conversion of encapsulated propagules into vigorous seedlings remains one of the important factors that hamper commercial production (Sharma et al. 2013). Adjusting the matrix composition of the beads will overcome the barriers that exist in storage and direct sowing. The hydrated calcium alginate-based or dried polyethylene glycol-based encapsulation will also provide the benefit of synthetic seed technology for long-term preservation of germplasms by cryopreservation (Ara et al. 2000).

The in vitro responses of wild beet species are not well studied yet. For instance, the somatic embryogenesis capability of these species is still unknown, whereas somatic embryo is the most widely used explant in synthetic seed production. Lack of in vitro studies along with frequently encountered difficulties may cause similar risks in the artificial seed production of wild *Beta* species.

8 Conclusion

Synthetic seeds have the potential to take part in the protection of endangered germplasms or to produce economic plants on commercial scales. Despite the importance of wild beet genotypes, synthetic seed technology for these species has

not yet been investigated. The development of in vitro protocols for somatic embryogenesis of these species is particularly important in the first step. The encapsulation procedure, as well as storage, should be examined for wild beet accessions.

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In Vitro Conservation Through Slow-Growth Storage

Ravishankar Chauhan, Vikram Singh, and Afaque Quraishi

Abstract In vitro approaches are valuable for the conservation of plant biodiversity that includes the preservation of genetic resources of vegetatively propagated species, threatened plant species, taxa with recalcitrant seed, elite genotypes, and genetically modified/engineered material. The mid-term conservation is usually achieved by reducing the growth of in vitro cultures through the application of minimal media and growth retardant or storage at low temperatures resulting in prolonged intervals between the subcultures. Moreover, the combinations of all these factors are also employed for slow-growth storage. The medium-term conservation strategies are consistently employed for a large number of plant species, including various threatened species, from tropical as well as temperate origin. For long-term conservation of plant species, cryopreservation (storage in liquid nitrogen at −196 °C) is commonly employed. However, the main difficulties associated with cryopreservation are the maintenance of in vitro cultures as the procedure is highly technical and expensive since it involves a huge amount of resources and labor. In vitro slow-growth storage, therefore, enables a possible solution for mid- to long-term conservation of plant materials in limited space and at reduced costs too. Slow-growth procedures allow clonal plant conservation for several months to years (depending upon the species) under aseptic conditions, requiring the infrequent successive transfers of the cultures.

Keywords Cold storage · Endangered species · Germplasm storage · Growth retardant · Minimal medium

R. Chauhan
National Center for Natural Resources, Pt. Ravishankar Shukla University, Raipur, India

V. Singh
School of Studies in Life Sciences, Pt. Ravishankar Shukla University, Raipur, India

A. Quraishi (✉)
School of Studies in Biotechnology, Pt. Ravishankar Shukla University, Raipur, India

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1 Introduction

The conventional method of plant germplasm conservation includes their maintenance as the whole plant in the field (Pathirana et al. 2016). Field maintenance of plant materials not only carries the risks of infections of viral, fungal, and bacterial diseases and insect pests but also includes losses due to the environmental disasters such as flood, earthquake, drought, fire, volcanic eruptions, etc., which has led to the erosion of valuable germplasm resources (Barba et al. 2008; Carimi et al. 2011). However, duplication of materials in different fields is an option but is a quite expensive approach. The major obstacles in in situ conservation practices are the requirement of larger space, the high cost of operation, complicated management, and risk of damage by both biotic and abiotic factors of the environment (Rao 2004). Therefore, risks involved in field maintenance have led us to search for secure, cost-effective, and efficient protocols for effective conservation of plant diversity. The plant genetic resources are conserved in the forms of seeds, bulbs, or tissue culture-derived propagules in various gene banks and termed as ex situ conservation (Paunescu 2009). Ex situ conservation is the maintenance of plant genetic resources under controlled conditions, i.e., away from their native habitats and cultivation in botanic gardens and nurseries and by seed storage or in gene banks through in vitro conservation (Dhillon and Saxena 2003; Paunescu 2009). In vitro approaches have two kinds of storage strategies: (1) active strategy which refers to short- to mid-term storage of samples and (2) base strategy referring to long-term preservation of materials (Linington 2003; Engelmann 2011). In gene banks, both the strategies are complementary to each other in which germplasms are stored in an environment free from vulnerable depletion by nature and by arthropods (Linington 2003; Li and Pritchard 2009). Both the strategies distribute disease-free plants, thus minimizing the cost of disease indexing (Lynch et al. 2007). Among these, the most suitable method suggested for long-term ex situ conservation of any species is storage of their seeds.

The species having orthodox seed form can be stored at a low temperature for extended periods by dehydrating down their moisture level (Roberts 1973). However, the conservation of other species and seed form is little problematic (Engelmann 2011), for example, the vegetatively propagated species that do not produce seeds. Similarly, recalcitrant seeds can't be dried sufficiently at the low moisture level with viability to let their storage at low temperatures (Roberts 1973). Moreover, the seeds of few species are generally highly heterozygous in nature and, therefore, unsuitable for the conservation purpose. Such species are thus chiefly maintained as clones (Engelmann 2011). Until now, most of the activities on ex situ conservation of plants have focused particularly on crops. However, conservation of wild and threatened plant species has also become an issue of concern. The statistics of the International Union for Conservation of Nature (IUCN) revealed that out of over 12,000 plant species, approx. 70% are in the threatened category and 19% are critically endangered (Trejgell et al. 2015). In addition, 28 species are extinct in the wild. In situ conservation strategy alone may not be sufficient to rescue the

threatened species (Sarasan 2010). In line, advancements in the biotechnology lead to the introduction of few novel categories of germplasm that includes clones obtained from elite genotypes, the cell lines with special attributes, and genetically transformed clones (Engelmann 1992). This new category is often of high added value and a bit problematic to produce (Engelmann 2011). The development of efficient techniques to ensure its safe preservation is, therefore, of paramount importance. A lot of efforts have been made to improve the quality and conservation methodologies by field gene banks and botanic gardens. However, clearly alternative approaches to plant genetic conservation are needed, and since the early 1970s, attention has turned to the possibilities offered by biotechnology, specifically in vitro culture system. Besides the conventional forms of protection of economically important and threatened species in the past decades, advancements in biotechnology and especially in the area of in vitro culture techniques led to the development of procedures that can be used as an excellent tool in plant conservation (Maryam et al. 2014). Plant tissue culture systems allow propagating plant material in an aseptic environment with high multiplication rates (Sharma et al. 2018). Disease-free clones can be obtained through meristem culture in combination with different therapies such as chemo-, thermo-, and electrotherapy, thus ensuring the production of disease-free stock materials and simplifying procedures for the germplasm exchange throughout the world (Singh et al. 2018). The miniaturization of explants allows reducing space requirements and, consequently, labor costs for the maintenance of plant germplasm. In vitro conservation protocols have been established for ample plant species, including a number of endangered species (Chauhan et al. 2016; Kamińska et al. 2016, 2018). In addition, another importance of ex situ conservation is that it is an internationally accepted strategy, as stated in the Global Strategy for Plant Conservation (UNEP 2002), and is frequently employed by a number of organizations known for biodiversity conservation (Sarasan et al. 2006). Plant tissue culture technique has been reported as an effective tool to conserve many plant species, especially of tropical origin (Engelmann 1991). For the short- and mid-term conservations, various techniques have been developed, which not only results in slow growth of the cultures but also prolongs the time interval between two subcultures (Cha-um and Kirdmanee 2007; Cordeiro et al. 2014).

2 Germplasm Storage Strategies

The maintenance of plant stocks or material under aseptic and adequate environmental conditions can be conducted using the two main approaches. The first one of these approaches is based on conserving material without disturbing its growth, i.e., successive transfer in a fresh medium, while the second one is based on conservation under slow-growth condition (Withers 1980; Engelmann 1991; Sarasan et al. 2006; Novikova et al. 2008). The shortcomings of a successive transfer are an increase in work expenses and the consumption of basic materials and nutrients (Cordeiro et al. 2014). It should also be taken into consideration that long-term subculture can be

followed by a decrease and/or the loss of the morphogenetic potential of the culture as well as by an increase in the probability of genetic changes during long-term subculturing (Joy et al. 1991; Bessembinder et al. 1993; Hao and Deng 2003). Furthermore, there is a risk of losing propagating material as a result of a human errors or microbial contamination in the process of subculture (Grout 1990); therefore, it is advisable to reduce frequent interventions during conservation.

With due regard for all these factors, in vitro culture under slow-growth conditions is supposed to be the most effective method of plant germplasm conservation. The use of this approach is aimed at slowing down the growth of cultures and prolonging the interval between two successive transfers (Cordeiro et al. 2014), as well as raising the degree of safety during the conservation of cultures as a result of a decrease in interferences in a culture system and the minimization of the risk of contamination during subculture (Grout 1990; Engelmann 2011). The success of the use of certain approach depends on numerous factors, such as the possibility of extending the time period between two successive transfer, how long the influence of a limiting factor lasts until the moment when that factor begins to negatively affect the culture, and how fast the regular developmental functions could be restored after reverting to standard culture conditions (Grout 1990). The essential condition for using slow-growth procedures is the study of vital capacities of various kinds of cultures and the stability/instability of the preserved material (Shibli et al. 2006; Rai et al. 2009).

3 Slow-Growth Storage Technique

Slow-growth storage (also known as mid-term conservation) is based on the reduction of the metabolic activity, i.e., the growth rate of in vitro cultures by maintaining them on modified growth medium or in altered culture conditions (Lambardi and Ozudogru 2013). The motto is to prolong the duration between two subcultures (depending on the species) of in vitro cultures without negatively affecting their regrowth potential. Reduction in the growth of in vitro cultures is generally achieved by modifying the culture medium and/or the culture conditions (Engelmann 1998, 2004). Among these approaches, the most widely applied practice is temperature reduction, which can popularly be coupled with a decrease in light intensity or incubation of culture in the dark condition (Engelmann 2011). A number of tropical species often show their susceptibility to low-temperature damage and hence can be stored at a comparatively higher temperature, which further depends on the cold sensitivity of the species (Engelmann 2011). And, to maintain in vitro culture, it should be subsequently subcultured under standard culture conditions to avoid contamination and/or deterioration of stock materials (Niino and Arizaga 2015). Manipulations of the culture medium may include dilution of mineral elements, reduction/enhancement of sugar concentration, changes in nature and/or concentration of plant growth regulators, and an addition of osmotically active compounds. Moreover, in a few cases, plant growth retardants were also applied (Acedo and

Arradaza 2012; Trejgell et al. 2015). Various parameters influence the efficiency of in vitro slow-growth storage procedures that includes the selection of explants, its chemical/physiological state during storage, the type of culture vessel, its volume, as well as the volume of a culture medium used for storage (Niino and Arizaga 2015, Engelmann 1991).

4 Low-Temperature Storage

The most extensively applied technique is temperature reduction, which can be pooled with a decrease in the light intensity or by maintaining the cultures in the dark conditions. Tropical and sub-tropical plant species are often cold-sensitive and have to be stored at higher temperatures, which depend on the cold sensitivity of the particular species. Potato in vitro plants can be stored at 7 °C without transfer for up to 18 months (Gopal and Chauhan 2010). Other species such as *Ananas* are much more cold-sensitive since the 66 accessions of *Ananas* shoot cultures have to be conserved at temperatures higher than 20 °C (Souza et al. 2004; Silva et al. 2016).

In vitro slow-growth storage procedures are being frequently used for medium-term conservation of a number of species, both from tropical and temperate origins, including crop and medicinal plants, e.g., *Coffea*, *Vitis*, *Musa*, and *Acorus* (Nassar 2003; Sajid et al. 2006; Kulkarni and Ganapathi 2009; Quraishi et al. 2017), and rare and endangered species (Thakur et al. 2015; Chauhan et al. 2016). However, if in vitro conservation appears as a simple and practical option for long-term conservation of various species and has extensive medium-term applications, its implementation still requires customization for any new species; continuous inputs are mandatory, and a question remains in regard to the clonal fidelity of the stored species. Moreover, it is not always possible to apply a single protocol for preserving genetically diverse species. For example, slow-growth storage experimentation performed with an in vitro collection of *Ananas* germplasm including 66 accessions revealed a huge variability in the response of the accessions to the storage conditions (Silva et al. 2016). Some of them showed somaclonal variation during storage, while others did not show any erosion.

Plant species storage at non-freezing low temperatures has been very successful (Koc et al. 2014). At lower temperature regime, the aging of the plant cells/tissues is slowed down but not completely stopped. Consequently, successive transfer of the plant material is necessary although very infrequently. Some examples where shoots/plants have been stored with different strategies of slow growth for various durations are listed in Table 1.

Preil and Hoffmann (1985) stored approx. 700 breeding lines of *Chrysanthemum* at 2–3 °C in the diffused light of 10–15 lux. At this condition, few of the lines survived up to 5 years, and the authors noticed that aeration of the cultures played a crucial role in storage. In the poor gas exchange conditions, the shoots became vitrified. Cold storage at a temperature of 10 °C in the diffuse light also induces vitrification of *Cheiranthera volubilis* shoots and, thus, reduces survival too (Williams and Taji 1987).

Table 1 In vitro slow-growth storage of plant species by employing three important factors, i.e., minimal medium, cold storage, and growth retardant either singly or in combination for mid-term conservation

Species	Explant/ organ	Kind of storage	Storage condition	Duration	Survival	References
Taraxacum pieninicum	Shoot tip	Minimal medium + cold storage + growth retardant	½ MS + sucrose (3%) + ABA (5 μM) at 10 °C	9 months	90%	Kamińska et al. (2016)
Chlorophytum borivilianum	Shoot	Minimal medium	MS + sucrose (6%) + BA (2 mg l^{-1}) + NAA (0.2 mg l^{-1})	4 months	100%	Chauhan et al. (2016)
Indigofera tinctoria	Nodal explants	Minimal medium	MS + IBA (0.5 mg l^{-1}) + mannitol (10 g l^{-1})	4 months	100%	Nair et al. (2016)
Senecio macrophyllus	Shoot tip	Minimal medium + growth retardant	½ MS + ABA (3.8 μM) + sucrose (3%) at 10 °C	9 months	100%	Trejgell et al. (2015)
Tetrastigma hemsleyanum	Shoot tip	Minimal medium + cold storage + growth retardant	½ MS + BA (0.3 mg l^{-1}) + sucrose (10 g l^{-1}) + maleic hydrazide (0.2 mg l^{-1}) at 8 °C	10 months	96%	Peng et al. (2015)
Vitis heyneana	Axillary buds	Minimal medium + growth retardant	MS + IBA (0.05 mg·l^{-1}) + IAA (0.1 mg l^{-1}) + ABA (0.5 mg l^{-1}) + mannitol (10 g l^{-1})	12 months	48%	Pan et al. (2014)
Pistacia lentiscus	Shoot tip	Cold storage	Basal MS in the dark condition at 4 °C	12 months	100%	Koc et al. (2014)
Castanea sativa	Shoot tip	Minimal medium + cold storage	WPM + BA (0.44 μM) at 8 °C	48 months	82%	Capuana and Lonardo (2013)
Glycyrrhiza glabra	Shoot apices	Minimal medium + cold storage + growth retardant	MS + BA (0.25 mg l^{-1}) + PEG (1 mg l^{-1}) + Ancymidol (5 mg l^{-1}) + ABA (0.1 mg l^{-1}) + mannitol (20 g l^{-1}) at 10 °C	6 months	100%	Srivastava et al. (2013)
Dioscorea alata	Node	Minimal medium	MS + mannitol (4%)	13 months	–	Acedo and Arradaza (2012)
Turbinicarpus	Shoot apices	Minimal medium + cold storage	MS + mannitol (30 g l^{-1}) + sorbitol (30 g l^{-1}) at 4 °C	12 months	–	Pérez-Molphe-Balch et al. (2012)

Solanum tuberosum	Node	Minimal medium	MS + sucrose (20 g l^{-1}) + sorbitol (40 g l^{-1})	18 months	58%	Gopal and Chauhan (2010)
Elettaria cardamomum	Node	Minimal medium	½ MS + BA (5 μM)	18 months	70%	Tyagi et al. (2009)
Saccharum spp.	Globular embryo	Minimal medium + cold storage	½ MS + sucrose (10 g l^{-1}) at 18 °C	8 months	–	Watt et al. (2009)
Dendranthema grandiflora	Node	Minimal medium	Modified hyponex medium + ½ MS + DMSO (2.5%) + sucrose (4%)	12 months	80%	Budiarto (2009)
Malus domestica and *Malus sieversii*	Shoot	Minimal medium + cold storage	MS + sucrose (3%) at 4 °C	21 months	–	Kovalchuk et al. (2009)
Fragaria x *ananassa*	Shoot	Minimal medium + cold storage	Knop's medium + sorbitol (0.2 M) at 4 °C	15 months	76%	Hassan and Bekheet (2008)
Veronica multifida ssp. *Capsellicarpa*	Node	Minimal medium	MS + mannitol (3 or 6%)	3 months	–	Holobiuc et al. (2008)
Drosophyllum lusitanicum	Node	Minimal medium + cold storage	MS + mannitol (2%) + sucrose (6%) + zeatin (0.91 μM) at 5 °C	8 months	100%	Gonçalves and Romano (2007)
Curcuma longa	Shoot	Minimal medium	MS + BA (2.5 mg l^{-1}) + isabgol (3.5%)	12 months	56%	Tyagi et al. (2007)
Allium sativum	Bulblet	Minimal medium + cold storage	MS + sorbitol (0.2 M) at 4 °C in dark	18 months	100%	Hasan et al. (2007)
Cedrus atlantica and *Cedrus libani*	Micro-cuttings	Minimal medium + growth retardant	MS + ABA (10 mg l^{-1})	6 months	83%	Renau-Morata et al. (2006)
Olea europaea	Shoot	Minimal medium + cold storage	OM medium at 4 °C in the dark condition	8 months	–	Rugini and Pesce (2006)
Vanilla spp.	Shoot buds	Minimal medium	½ MS + sucrose (1.5%) + mannitol (1.5%)	12 months	90%	Divakaran et al. (2006)
Dierama luteoalbidum	Corm	Minimal medium + growth retardant	MS + paclobutrazol (5–10 mg l^{-1})	6 months	100%	Madubanya et al. (2006)

(continued)

Table 1 (continued)

Species	Explant/ organ	Kind of storage	Storage condition	Duration	Survival	References
Vriesea reitzii	Shoot	Minimal medium	MS + NAA (2 µM) + BA (4 µM)	4 months	100%	Filho et al. (2005)
Garcinia indica	Shoot	Minimal medium	½ MS + BA (5 µM) + sucrose (3%)	11 month	95%	Malik et al. (2005)
Plumbago indica	Nodal segments	Minimal medium	MS + mannitol (20 g l^{-1})	8 months	100%	Charoensub and Phansiri (2004)
Melia azedarach	Apical mer-istem-tip	Minimal medium + cold storage	¼ MS + BA (0.5 µM) at 4 °C in dark	12 months	67%	Scocchi and Mroginski (2004)
Ananas comosus	Shoot	Minimal medium + growth retardant	MS + paclobutrazol (0.5 mg l^{-1})	3 months	100%	Canto et al. (2004)
Ipsea malabarica	Shoot	Minimal medium	½ MS only	27 months	100%	Martin and Pradeep (2003)
Mentha spp.	Apical and nodal explants	Cold storage	MS only at 2 °C	6 months	–	Islam et al. (2003)
Malus pumila cv Gala	Shoot tip	Minimal medium + cold storage	MT + BA (0.5 mg l^{-1}) + NAA (0.05 mg l^{-1}) + sucrose (2%) + mannitol (2%) at 4 °C	12 months	100%	Hao and Deng (2003)
Phoenix dactylifera	Shoot bud	Cold storage	MS only in darkness at 5 °C	12 months	70%	Bekheet et al. (2002)
Solanum tuberosum	Node	Minimal medium + cold storage + growth retardant	MS + sucrose (6%) + Ancymidol (25 µM) at 6 °C	16 months	92%	Sarkar et al. (2001)
Ensete ventricosum	Shoot	Minimal medium + cold storage	MS + BA (10 µM) + mannitol (1%) at 15 °C	12 months	86%	Negash et al. (2001)
Malus pumila cv Moscatella	Apical/node	Cold storage	½ MS in darkness at 4 °C	12 months	100%	Negri et al. 2000

Malus pumila cv Starkspur red	Apical/ node	Cold storage	½ MS in darkness at 4 °C	18 months	90%	Negri et al. (2000)
Musa spp.	Shoot tips	Cold storage	MS only at 17 °C	15 months	66%	Pedroso de Oliveira et al. (2000)
Solanum tuberosum	Shoot	Cold storage	MS only at 4 °C in red light illumination	3 months	–	Pruski et al. (2000)
Prunus virginiana	Shoot	Cold storage	MS only at 4 °C in red light illumination	3 months	–	Pruski et al. (2000)
Quercus suber	Shoot	Cold storage	GD medium at 5 °C in the dark	24 months	50%	Romano and Martins-Loução (1999)
Coffea arabica	Zygotic embryo	Minimal medium + growth retardant	MS + ABA (18.9 or 37.8 μM)	24 months	74%	Naidu and Sreenath (1999)
Solanum tuberosum	Node	Minimal medium + cold storage	MS + mannitol (2%) at 6 °C	16 month	88–100%	Sarkar et al. (1999)
Solanum tuberosum	Node	Minimal medium + cold storage	MS + sucrose (40 g l^{-1}) + mannitol (20 g l^{-1}) at 6 °C	30 months	83%	Sarkar and Naik (1998)
Solanum tuberosum	Node	Minimal medium + cold storage	MS + mannitol (4%) + acetylsalicylic acid (100 μM) at 8 °C	12 months	90%	Lopez-Delgado et al. (1998)
Lilium hybrid	Scale bulblet	Minimal medium + cold storage	¼ MS + sucrose (9%) at 2 °C	28 months	73–90%	Bonnier and van Tuyl (1997)
Miscanthus x *ogiformis*	Shoot	Minimal medium + cold storage	½ SH medium + NAA (0.1 μM) at 8–16 °C	6 months	95–100%	Hansen and Kristiansen (1997)
Coffea spp.	Shoot tip	Minimal medium	MS + BA (1.3 μM)	36 months	–	Dussert et al. (1997)
Xanthosoma spp.	Shoot	Minimal medium + cold storage	MS + mannitol (3%) at 13 °C	24 months	–	Zandvoort et al. (1994)

(continued)

Table 1 (continued)

Species	Explant/ organ	Kind of storage	Storage condition	Duration	Survival	References
Colocasia esculenta	Single shoot	Minimal medium + cold storage	MS + BA (10 µM) + mannitol (1–2%) at 9 °C	96 months	90%	Bessembinder et al. (1993)
Saccharum spp.	Apical meristem	Minimal medium + cold storage	½ MS + sucrose (1%) at18°C	12 months	–	Taylor and Dukic (1993)
Cocos nucifera	Zygotic embryo	Minimal medium	MS + sodium ascorbate (100 mg l^{-1}) + sucrose (1.5%)	12 months	51%	Assy-Bah and Engelmann (1993)
Populus alba x *Populus grandidentata*	Shoot	Minimal medium + cold storage	MS + BA (1.33 µM) at 4 °C	60 months	25%	Son et al. (1991)
Musa acuminata	Meristem tip	Minimal medium + cold storage	SM + ribose (3%) at 17 °C	24 months	67%	Ko et al. (1991)
Ipomoea batatas	Axillary buds	Minimal medium + growth retardant	MS + ABA 10 (mg l^{-1})	12 months	20–80%	Jarret and Gawel (1991)

ABA abscisic acid, *BA* 6-benzylaminopurine, *DMSO* dimethyl sulfoxide, *GD* Gresshoff and Doy (1972) medium, *IBA* indole-3-butyric acid, *MS* Murashige and Skoog (1962), *MT* Murashige and Tucker (1969) medium, *NAA* naphthalene acetic acid, *OM* Rugini (1984) medium, *SH* Schenk and Hildebrandt (1972) medium, *SM* Smith and Murashige (1970) medium, *WPM* woody plant media (McCown and Lloyd 1981)

The double-node cuttings of *Drosophyllum lusitanicum* could be kept alive for 8 months at 5 °C in growth-limiting condition (Gonçalves and Romano 2007). Low-temperature storage has been applied with most promising results to in vitro shoot/plantlet cultures and less successfully to undifferentiated cell cultures. Slow-growth can retard the loss of totipotency of cultured cells/tissue and the ability to synthesize secondary metabolites too in callus cultures stored for relatively short periods (Seitz 1987). The storage temperature usually depends on the sensitivity of the species. Whereas for temperate species it ranges from 5 to 9 °C, for tropical species, it is often much higher (Kulkarni and Ganapathi 2009). Shoot tips of *Actinidia* spp. could be maintained at 8 °C for 52 weeks with 100% survival (Monette 1987). The shoots retrieved after a storage period appeared normal in respect to growth and proliferation rates. Similarly, the cultures of *Colocasia esculenta*, another tropical species, conserved for 3 years at 9 °C (Zandvoort and Staritsky 1986). On the contrary, *Musa* cell suspension cultures were not able to tolerate temperatures lower than 15 °C (Kulkarni and Ganapathi 2009). Few banana cultivars that were stored below 15 °C suffered damage within 3 months (Withers and Williams 1986). At 15 °C some of the accessions of banana survived up to 17 months with a viability of 92%, but in others, viability was sharply reduced to 50% within 13 months (Withers and Williams 1986). According to Watt et al. (2009), the best condition for the storage of globular somatic embryos of *Saccharum* spp. is to place them on ½-strength MS medium (Murashige and Skoog 1962) supplemented with only 10 g L^{-1} of sucrose and incubate at 18 or 24 °C. *Saccharum* spp. stored under these conditions for 8 months showed approx. 80% survival, and most of the plants appeared normal. Similarly, cassava plantlets must be stored at temperatures higher than 20 °C (Roca et al. 1984). The low-temperature storage protocols of maintaining in vitro cultures hold great promise in the nursery industries (Preil and Hoffmann 1985). During the periods of low demand for a particular species or variety for which potential markets exist in the future, the in vitro cultures may be efficiently shelved in normal refrigerators and the time and, consequently, money required to maintain them by successive transfer or restarting fresh cultures saved. This methodology may also apply to research stocks for further experimentation. However, one of the expected limitations in low-temperature storage of plant germplasm may be the gradual habituation of some materials to slow-growth conditions (Withers 1991).

5 Minimal Medium

By modifying the medium composition usually by reducing the sugar content, minerals, growth regulators, or osmotic agents such as sorbitol and mannitol, inhibition of cell division can be achieved, which significantly limits both callus formation and shoot development (Shibli et al. 2006; Lambardi and Ozudogru 2013). In this context, the in vitro slow-growth storage of *Elettaria cardamomum* was achieved on the half-strength MS, fortified with 30 g L^{-1} of sucrose, of which

about 70% of the cultures survived up to 18 months at 25 °C (Tyagi et al. 2009). A regrowth potential of 96% was obtained by culturing nodal explants excised from 18-month-old conserved cultures of *E. cardamomum*.

Photinia sp. micro-shoots were able to store at 4 °C and up to 15 months in a combination of sucrose and mannitol 15 g L^{-1} each containing QL medium (Quoirin and Lepoivre 1977) with more than 90% of survival rate (Akdemir et al. 2010). The stored materials were further recovered and found to proliferate normally in 1 mg L^{-1} BA supplemented QL medium. The micro-plants of six genotypes of *Solanum tuberosum* could be preserved for up to 12 months, without any phenotypic abnormalities, and had enough nodes for further subculturing. The conservation was conducted at 24 °C in the MS medium supplemented with 20 g L^{-1} sucrose along with 40 g L^{-1} sorbitol at a photoperiod of 16/8 h, in which the survival rate was 77.8% (Gopal et al. 2002). This approach was an effective alternative to low-temperature (6–8 °C) storage, especially for the species of tropical and sub-tropical origins, where summer temperature may reach up to 45–50 °C (Gopal et al. 2002).

The effects of osmotic doses along with different temperature regimes were found efficient for tuber- or bulb-producing species. MS medium, comprising 3% (w/v) sucrose, 4% (w/v) sorbitol, and 1 mg L^{-1} ancymidol, was seen to be the best suited for slow-growth storage of in vitro cultured crowns of *Asparagus officinalis* (Fletcher 1994), in which crowns were stored at 6 °C for 16 months and were regrown with 100% survival. Similarly, Bonnier and van Tuyl (1997) successfully stored the in vitro bulblet of *Lilium* spp. for a period of 28 months at 25 °C on ¼-strength MS medium supplemented with 9% (w/v) sucrose. Afterward, these cultures were successfully regenerated with a survival rate of 92%.

Further, the combined effects of sucrose, mannitol, and photoperiod were assessed at 6 °C of temperature for the conservation of micro-shoots of *S. tuberosum* by Sarkar and Naik (1998). Their slow-growth media were comprised of 30, 40, 50, 60, 70, or 80 g L^{-1} sucrose along with 20, 40, or 60 g L^{-1} of mannitol. Over 30 months of storage, sucrose alone did not improve the viability of these cultures. However, the addition of 20 g L^{-1} of mannitol in the storage medium increased the survival rate (83%) of micro-shoots. Further, in order to in vitro conserve the *Saccharum officinarum* germplasms, lateral buds onto the MS medium with an osmoticum were screened (Sarwar and Siddiqui 2004). In the 2% mannitol (w/v)-supplemented MS medium, the lateral buds were healthy up to 165 days and with 75% survival rate, while the cultures with 3% (w/v) mannitol showed 100% survival up to 105 days only, at 17 °C of temperature; conducted study also suggested that low temperature (10 °C) was unfavorable for in vitro storage of *S. officinarum* under both light and dark conditions.

The in vitro shoot tips of *Vanilla planifolia* could successfully be maintained for more than 1 year, without subculturing, on the MS medium supplemented with 15 g L^{-1} each of sucrose and mannitol, at 22 °C and with 90% recovery (Divakaran et al. 2006). After few years of this report, the in vitro shoots of *Saccharum* sp. were successfully stored in the ½-strength MS medium amended with 30 g L^{-1} sorbitol, at both 18 and 24 °C for a duration of 8 months (Watt et al. 2009). The highest survival percentage and shoot regrowth (90%) were observed in cultures stored at 18 °C.

Studies pertaining to in vitro preservations of *Dianthus spiculifolius* and *D. tenuifolius* were performed (Mitoi et al. 2009), in which addition of 0.16 and 0.32 M mannitol in the MS medium, in combination with vitamin of B5 medium (Gamborg et al. 1968), was found to be most suitable for their conservation and regeneration after 6 months of storage at 25 °C. The addition of mannitol (58.4 mM) in the MS medium was found best for 7 months' storage at 5 °C of *Prunus* sp. with a survival rate of 100% (Marino et al. 2010). In the case of *Podophyllum peltatum*, storage of micro-shoots at 10 and 25 °C of temperatures revealed 100% survival after the addition of mannitol (2%, w/v) or sorbitol (2%, w/v) to the MS medium (Lata et al. 2010). However, a negative impact of both the osmoticum, in terms of shoot proliferation rate, was there when stored micro-shoots were cultured on a recovery medium (Lata et al. 2010). Likewise, the shoot tips of *Pyrus* sp. could be stored successfully on the MS medium containing 2.5% (w/v) mannitol, with highest survival (63.41%) and regeneration (58.81%) potentials at 25 °C (Ahmed and Anjum 2010). The efficiencies of sucrose, mannitol, or sorbitol, in a series of concentrations (3%, 6%, 9%, or 12%, w/v), were assessed for storage of *Stevia rebaudiana* micro-shoots (Shatnawi et al. 2011). Afterward, a dose of 3–9% (w/v) sucrose was found to favor higher survival (94.6%) of micro-shoots even after their storage for 32 weeks. However, under dark storage, the survival rate of these cultures was reduced significantly. The micro-shoots of two *Pistacia vera* cultivars were maintained at 4 °C in the dark conditions for 12 months in the MS medium containing 2% (w/v) of mannitol (Akdemir et al. 2013). More than 90% of proliferation in the micro-shoots of both the cultivars was observed after 12 months of storage.

6 Application of Growth Retardant

Growth retardants are natural/synthetic chemical compounds that can be applied in the culture medium to change vital processes by modifying hormonal balance in a plant in vitro (Espindula et al. 2009). Growth retardants act as signalling compounds in the regulation of plant growth and development. They typically bind to receivers in the plant and induce a series of cell changes that can affect the initiation/modification of tissue development (Espindula et al. 2009). The augmentation of plant growth inhibitors in the culture medium is also a significant measure to retard cell growth; such inhibitors include abscisic acid (ABA), maleic hydrazide, paclobutrazol, and few others (Renau-Morata et al. 2006; Sharma et al. 2012; Trejgell et al. 2015).

Kovalchuk et al. (2009) successfully conserved the micro-shoots of *Malus domestica* for 21 months in the MS medium augmented with 1 mg L^{-1} of ABA at 4 °C, while *Glycyrrhiza glabra* shoot apices cultures responded best for storage up to 6 months, when incubated at 10 °C under a dark condition in 5 mg L^{-1} ancymidol, 0.1 mg L^{-1} ABA, and 1 mg L^{-1} polyethylene glycol (Srivastava et al. 2013). Addition of 9.5 μM ABA and 1.5% (w/v) each of sucrose and sorbitol could enhance survival and proliferation of 9-month-old micro-shoots of *Senecio macrophyllus*

during their re-culture in optimal conditions, compared to those cultures stored on the MS medium lacking ABA (Trejgell et al. 2015).

Recently, the subculture duration of *Tetrastigma hemsleyanum* micro-shoots was significantly prolonged up to 10 months, by using 0.2 mg L^{-1} of maleic hydrazide 8 °C with 8/16 h photoperiod (Peng et al. 2015). In this study, the addition of growth retardants such as ABA, chlormequat, and paclobutrazol did not improve *T. hemsleyanum* micro-shoot survival at both 8 and 25 °C of temperatures.

7 Slow-Growth Storage of Elite Tree Species

In vitro propagation technique plays a key role in increasing the production of woody plants and the re-establishment of threatened plant germplasms (Quraishi 2013). However, those techniques involve periodic subculturing of cultures to change the gaseous state of the vessels and to refresh the components of a medium (Ozden-Tokatli et al. 2010). Plant biodiversity comprises ample old mature tree diversity having various featuring characteristics such as *Ginkgo biloba*: the living fossil or the *Taxus* trees famous for their anti-cancer bioactive compound. For the exploitation and conservation of such elite clones, slow-growth storage can be useful as few hard-wood species efficiently conserved through these techniques.

Pistacia lentiscus can be efficiently stored in 6 months at 4 °C in the dark (Koc et al. 2014). Further, the response of mannitol was examined for mid-term conservation of *Eucalyptus ventricosum* (Negash et al. 2001). *E. ventricosum* could be effectively conserved up to 6 months' duration at 15 °C. Similarly, in vitro shoot tips of *Malus pumila* were stored at 4 °C on the Murashige and Tucker (1969) medium fortified with 2% (w/v) each of sucrose and mannitol, in which all the shoot tips were able to survive up to 12 months of storage, revealing 100% recovery (Hao and Deng 2003). The mid-term storage of *Eucalyptus grandis* shoot cultures was achieved for up to 10 months by the addition of 10 mg L^{-1} of ABA at 25 °C of temperature (Watt et al. 2000). In this approach, reductions in the light intensity and addition of mannitol to the MS medium were found to be less effective for the conservation of *E. grandis*. Very recently, 45 or 60 g L^{-1} of sucrose fortified DKW medium (Driver and Kuniyuki 1984) was found to be suitable for in vitro conservation of *Prunus avium* shoots (Ozudogru et al. 2017). At 4 °C and under dark condition, these cultures survived up to 16 months of time. However, the inclusion of mannitol in the storage medium did not reveal any significant impact on shoot quality of *P. avium*.

8 Conclusion

For long-term storage, cryopreservation, i.e., storage at an ultra-low temperature, usually that of liquid nitrogen (−196 °C), is the only method. At this temperature, all cellular divisions and metabolic processes are stopped. The plant material can thus

be stored without alteration or modification for a theoretically unlimited period of time (Barraco et al. 2013). However, the main difficulties associated with long-term maintenance of in vitro cultures are that the procedure is a bit problematic, highly technical, and expensive as it involves the huge amount of resources and labor (Rao 2004; Capuana and Lonardo 2013). In vitro slow growth, therefore, represents a possible solution for mid- to long-term storage of plant materials in limited space and at reduced costs. Furthermore, slow-growth procedures allow clonal plant conservation for several months to years (depending upon the species) under aseptic conditions, requiring infrequent subculturing (Cha-um and Kirdmanee 2007). Of the numerous methodologies tried for short-/medium-term conservation of germplasms, lowering the temperature regime of culture has been most accepted so far and is being used for routine maintenance of germplasm of a range of plant species (Withers 1991).

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Synthetic Seeds: Prospects and Advances in Cryopreservation

Débora de Oliveira Prudente, Lucas Batista de Souza, and Renato Paiva

Abstract Long-term storage of synthetic seeds can be accomplished using cryopreservation techniques. Cryopreservation allows the viability of encapsulated plant material to be conserved and maintained over a long period without modifications or genetic changes because the material is exposed to ultralow temperatures in liquid nitrogen (−196 °C), which decreases or even halts cellular metabolism. Cryopreservation has been found to be practical and efficient for the conservation of many species due to the small volume of material needed for storage, the simplification of transportation procedures and the minimal maintenance required compared to conventional storage methods. The main cryopreservation techniques applied to synthetic seeds are encapsulation-dehydration and encapsulation-vitrification. These techniques have been shown to be highly applicable for small explants that are sensitive to the conventional cryopreservation process, such as meristems and somatic embryos. However, the success of cryopreservation techniques for synthetic seeds depends on the type of encapsulated explant, on the capsule constitution and consistency and on research on the different cryopreservation stages in order to optimize the survival and regeneration of the plant material. Therefore, the present chapter is based on studies of the different stages of cryopreservation related to encapsulation techniques developed over time and on the major advances and innovations in cryopreservation.

Keywords Long-term storage · Plant germplasm · Cryopreservation · Encapsulation-dehydration · Encapsulation-vitrification

1 Introduction

The problems associated with the difficulty of germinating and storing recalcitrant species can be minimized through a plant tissue culture technique. This technique consists of encapsulating somatic embryos, shoot apices and lateral buds, among

D. de Oliveira Prudente (✉) · L. B. de Souza · R. Paiva
Universidade Federal de Lavras, Lavras, Brazil

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other types of explants, in a matrix composed of gelling substances, giving rise to synthetic seeds (Neal Stewart 2016).

The use of synthetic seed technology has increased significantly in recent years, as it is a rapid and efficient asexual propagation method (Rai et al. 2009). Encapsulation also facilitates the exchange of genetic material and has been gaining prominence in germplasm conservation (Matsumoto 2017).

For the short term, synthetic seeds are stored under aseptic conditions and reduced to a temperature of 4 °C for a maximum of 12 months (Javed et al. 2017). For long-term storage, cryopreservation is recommended because it is a technique for storing living biological material at an ultralow temperature (−196 °C). Under these conditions, the occurrence of thermally driven metabolic reactions is low or nonexistent, guaranteeing the viability of the biological material without modification or genetic alteration over time (Engelmann 2011).

Cryopreservation can be applied for the conservation of different types of explants such as protoplasts, cell suspensions, embryogenic calli, shoot apices, lateral buds, seeds, zygotic embryos and somatic embryos (Benson 2008). Cryogenic collections, i.e., the set of cryopreserved samples from the various types of explants, are kept in small spaces, remaining protected from contamination and requiring minimal maintenance (Engelmann 2004; Wyse et al. 2018). In addition, the cost of long-term storage in a cryogenic bank is lower than that of other available genetic material conservation systems, such as field germplasm banks and slow-growth in vitro conservation (Dulloo et al. 2009; Kushnarenko et al. 2018).

The main cryopreservation techniques applied to synthetic seeds include encapsulation-dehydration and encapsulation-vitrification. In encapsulation-dehydration, the encapsulated explants undergo physical dehydration, which rapidly removes large volumes of water from the capsules (Panis et al. 2005). Encapsulation-vitrification is based on osmotic dehydration, which is strictly dependent on the increased viscosity of the dehydrating solution due to the high concentration of solutes in solution (Sakai and Engelmann 2007).

In cryopreservation protocols, it is essential to avoid ice crystal formation in the intracellular environment (Mazur 1984). Increasing viscosity reduces ice crystal formation in plant cells, but the vitrification state of the cytoplasm is unstable, especially during the capsule reheating step, which is necessary for thawing the material to be regenerated. At this stage, large ice crystals may form when the cytoplasm begins to pass from the vitrified to the liquid state, which is characterized by recrystallization (Mazur 1984). To avoid recrystallization, the material must be removed from liquid nitrogen and rapidly reheated (Benson 2008). The vitreous state may be induced by using cryoprotective solutions or dehydration techniques (Sakai et al. 2008).

Ice crystal formation can be prevented by dehydrating the capsules, without affecting the explants enveloped by these capsules (Engelmann 2011). Encapsulation is mainly required for small explants, such as meristems, buds and somatic embryos. In addition, encapsulation facilitates handling and protects structures that will be immersed in liquid nitrogen against possible damage caused by cooling, making the explants more resistant to potentially lethal treatments (Paulet et al. 1993; Bachiri et al. 1995; Matsumoto 2017).

In this context, it is evident that cryopreservation for conserving synthetic seeds has high applicability. However, studies on capsule constitution and consistency, in vitro culture conditions and adjustments to the different steps of synthetic seed cryopreservation techniques are necessary to ensure high rates of survival and regeneration of the plant material.

2 Modification of Cryopreservation Protocols of Synthetic Seeds: Encapsulation-Dehydration and Encapsulation-Vitrification

2.1 Type of Explants

The choice of the appropriate cryopreservation protocol for each type of explant depends primarily on the physiological state of the cells and tissues that will be cryopreserved. Explants from young and healthy plants should be prioritized because they exhibit less oxidation throughout the process, although mature tissues are also frequently used (Gulati 2018).

In general, small structures are more appropriate for cryopreservation, since their dehydration and freezing occur more rapidly and uniformly and their cells have reduced vacuoles and dense cytoplasm, resulting in decreased cell volume retraction (Volk and Caspersen 2007).

Explants from species that propagate vegetatively have great applicability for the conservation of selected varieties with characteristics of interest (Engelmann 2004). However, this type of plant material tends to be more sensitive to cellular dehydration, since meristems, shoot apices, axillary buds and somatic embryos have high water content. In these cases, it is necessary to prepare the plant material before cooling with increased care to maintain the integrity of the cellular structure to be cryopreserved and intensify the membrane protective mechanisms against excessive dehydration (Prudente and Paiva 2017; Reed 2018).

The encapsulation of explants facilitates tolerance to solutions with high sucrose concentrations and protects against dehydration in a low-moisture environment, which would be highly detrimental or lethal for nonencapsulated samples (Reed 2018). This technique has been successfully applied to a wide variety of plant species from temperate and tropical climates, encompassing different types of explants, as listed in Table 1.

2.2 Preconditioning

The first step of cryopreservation in some protocols is called the preculture phase. It consists of a preparatory phase where explants are subjected to the conditions

Table 1 List of plant species cryopreserved using the encapsulation-dehydration and encapsulation-vitrification techniques

Species	Encapsulation-dehydration	Encapsulation-vitrification
African violet (*Saintpaulia ionantha*)	ST (Moges et al. 2004)	ST (Moges et al. 2004)
Air potato (*Dioscorea bulbifera*)	–	C (Ming-Hua and Sen-Rong 2010)
Ajania (*Ajania pacifica*)	ST (Kulus and Abratowska 2017)	–
Alder (*Alnus glutinosa*)	–	–
Algarve (*Plantago algarbiensis*)	NS (Coelho et al. 2014)	–
Apple (*Malus spp.*)	R (Bettoni et al. 2018) ST (Niino and Sakai 1992; Li et al. 2015; Wang et al. 2018a, b)	ST (Wang et al. 2018a)
Arabidopsis (*Arabidopsis thaliana*)	ST (Bonnart and Volk 2010)	–
Asian madder (*Rubia akane*)	–	HR (Shin et al. 2014)
Asparagus (*Asparagus officinalis*)	R (Carmona-Martin et al. 2018)	ST (Jeon et al. 2015)
Avocado (*Persea americana*)	–	–
Banana (*Musa spp.*)	M (Panis et al. 1996)	–
Beech (*Fagus sylvatica*)	–	–
Blueberry (*Vaccinium corymbosum*)	ST (Uchendu and Reed 2008; Kami et al. 2009)	ST (Kami et al. 2009)
Blushred Rabdosia (*Rabdosia rubescens*)	ST (Ai et al. 2012)	–
Cassava (*Manihot esculenta*)	ST (Engelmann and Takagi 2000)	ST (Charoensub et al. 2004)
Chrysanthemum (*Chrysanthemum morifolium*)	ST (Kulus et al. 2018a, b)	ST (Lia et al. 2019)
Citrus (*Citrus* spp.)	SE (Gonzalez-Arnao et al. 2000) O (Gonzalez-Arnao et al. 2003) ST (Rohini et al. 2016)	EC (Souza et al. 2017)
Cocoa (*Theobroma cacao*)	–	–
Common box (*Buxus hyrcana*)	ST (Kaviani and Negandar 2017)	–
Cranberry (*Vaccinium macrocarpon*)	ST (Uchendu and Reed 2008; Kami et al. 2009)	ST (Kami et al. 2009)
Date palm (*Phoenix dactylifera*)	EC (Subaih et al. 2007) C (Al-Qurainy et al. 2017)	EC (Subaih et al. 2007) C (Al-Qurainy et al. 2017)
Eucalyptus (*Eucalyptus* spp.)	ST (Kaya et al. 2013)	–
Felty germander (*Teucrium polium*)	ST (Rabba'a et al. 2012)	–

(continued)

Table 1 (continued)

Species	Encapsulation-dehydration	Encapsulation-vitrification
Fern (*Osmunda regalis*)	–	G (Makowski et al. 2016)
Garlic (*Allium sativum*)	NS (Lynch et al. 2016)	–
Gentian (*Gentiana* spp.)	–	ST (Tanaka et al. 2004)
Grapevine (*Vitis* spp.)	ST (Bi et al. 2017)	ST (Bi et al. 2017)
Guinea hen weed (*Petiveria alliacea*)	SE (Pettinelli et al. 2017)	–
Hamlin sweet orange (*Citrus sinensis*)	–	–
Himalayan mulberry (*Morus laevigata*)	NS (Choudhary et al. 2018)	–
Hladnikia (*Hladnikia pastinacifolia*)	ST (Ciringer et al. 2018)	ST (Ciringer et al. 2018)
Horseradish (*Armoracia rusticana*)	ST (Phunchindawan et al. 1997)	ST (Phunchindawan et al. 1997)
Kiwifruit (*Actinidia chinensis*)	–	–
Maize (*Zea mays*)	ZE (Thobunluepop et al. 2009)	–
Mandevilla (*Mandevilla moricandiana*)	NS (Cordeiro et al. 2014)	–
Mangaba tree (*Hancornia speciosa*)	–	–
Mint (*Mentha* × *piperita*)	ST (Martín et al. 2015; Gonzalez-Benito et al. 2016)	–
Oak (*Quercus robur*)	–	–
Olive (*Olea europaea*)	SE (Shibli and Al-Juboory 2000)	SE (Shibli and Al-Juboory 2000)
Orchids (Orchidaceae)	P, ST (Popova et al. 2016)	–
Papaya (*Carica papaya*)	–	–
Passion fruit (*Passiflora* spp.)	–	ST (Garcia et al. 2011; Merhy et al. 2014)
Pineapple (*Ananas comosus*)	ST (Gonzalez-Arnao et al. 1998)	ST (Gamez-Pastrana et al. 2004)
Potato (*Solanum tuberosum*)	ST (Zarghami et al. 2008)	M, B (Hirai and Sakai 1999; Li et al. 2017)
Red raspberry (*Rubus idaeus*)	–	–
Redwood (*Sequoia sempervirens*)	ST (Halmagyi and Deliu 2011)	B (Ozudogru et al. 2011)
Rose (*Rosa hybrida*)	IFEs (Mubbarakh et al. 2014)	–
Shih (*Artemisia herba-alba*)	ST (Sharaf et al. 2012)	ST (Sharaf et al. 2012)
Small murici (*Byrsonima intermedia*)	–	–
Soybean (*Glycine max*)	–	–

(continued)

Table 1 (continued)

Species	Encapsulation-dehydration	Encapsulation-vitrification
St John's wort (*Hypericum* spp.)	M (Brunakova and Cellarova 2016)	–
Strawberry (*Fragaria* × *ananassa*)	ST (Clavero-Ramirez et al. 2005)	–
Sugarcane (*Saccharum officinarum*)	ST (Barraco et al. 2011; Rafique et al. 2016)	ST (Kaya and Souza 2017)
Sweet potato (*Ipomoea batatas*)	ST (Agbidinoukoun et al. 2018)	–
Thyme (*Thymus* spp.)	–	ST (Ozudogru and Kaya 2012)
Tobacco (*Nicotiana tabacum*)	–	ST (Uchendu et al. 2013)
Todsen's pennyroyal (*Hedeoma todsenii*)	ST (Pence et al. 2017)	ST (Pence et al. 2017)
Tomato (*Solanum lycopersicum*)	ST (Al-Abdallat et al. 2017)	–
Wasabi (*Wasabia japonica*)	–	M (Matsumoto et al. 1995)
Wheat (*Triticum aestivum*)	S (le Roux et al. 2016)	S (le Roux et al. 2016)
Yam (*Dioscorea alata* and *D. cayenensis*)	ST (Uchendu and Keller 2016)	–

C calli, *EC* embryogenic cultures, *M* meristems, *NS* nodal segments, *P* protocorms, *R* roots, *S* seeds, *SE* somatic embryos, *ZE* zygotic embryos, *ST* shoot tips, *G* gametophytes, *B* buds, *IFEs* in vitro fragmented explants

necessary for tolerance to critical stages such as cellular dehydration (Mathew et al. 2018). After water removal, the solutes become more concentrated in the intracellular environment, which can result in an increased rate of destructive chemical reactions, as the ionic potential and pH of the intracellular solution will change, modifying the cell's metabolic status (Kramer and Boyer 1995; Prudente and Paiva 2017).

The success of a cryopreservation protocol therefore depends on dehydrating the cell to a water content low enough to prevent intracellular crystal formation but not so low as to cause injury due to dehydration.

The hydroxyl groups of the sugars will bind via hydrogen bonds to the hydrophilic groups of the polar heads of phospholipids and of the proteins in the cell membrane bilayer to maintain the hydrophilic structures in their hydrated conformation even after water has been removed (Crowe et al. 1987; Lynch et al. 2011).

In this phase, progressively increasing the concentration of sugars and/or supplementing the culture medium with cryoprotective compounds, such as antioxidants, alcohols, amines and other substances, for a given period (hours to weeks, depending on the culture) are essential procedures for the survival of the plant material (Table 2).

Table 2 Cryoprotective compounds added to the preculture medium

Cryoprotectants	References
Antioxidants	
Ascorbic acid	Wang et al. (2005), Gonzalez-Benito et al. (2016)
Citric acid	Wang et al. (2005)
Glutathione	Gonzalez-Benito et al. (2016)
Lipoic acid	Uchendu et al. (2010)
Melatonin	Ren et al. (2014), Uchendu et al. (2014)
Salicylic acid	Pathirana et al. (2016), Volk et al. (2018)
Tocopherol	Gonzalez-Benito et al. (2016)
Alcohols and derivatives	
Ethylene glycol	Paul et al. (2000), Kumar and Sharma (2005), Chandrabalan et al. (2011)
Glycerol	Bi et al. (2018), Chen et al. (2018), Salama et al. (2018)
Propylene glycol	Fabian et al. (2008)
Sugars and sugar alcohols	
Fructose	Sipen et al. (2011)
Galactose	Dumet et al. (1994)
Glucose	Suzuki et al. (2008), Sipen et al. (2011)
Mannitol	Van Eck and Keen (2009), Sipen et al. (2011)
Raffinose	Bustam et al. (2016)
Sorbitol	Fatima et al. (2009)
Sucrose	Sipen et al. (2011), Prudente et al. (2017), Bi et al. (2018), Ciringer et al. (2018)
Trehalose	Sipen et al. (2011)
Polymers	
Polyethylene glycol (PEG)	Paques et al. (1996)
Polyvinylpyrrolidone (PVP)	Wang et al. (2005), Uchendu et al. (2010)
Sulphoxides and amides	
Dimethyl sulphoxide	Suranthran et al. (2012), Zhang et al. (2014), Lineros et al. (2018)
Amines	
Betaine	Uchendu et al. (2010)
Glutamine	Ryynahen and Haggman (1999), Uchendu et al. (2010)
Glycine betaine	Uchendu et al. (2010), Prudente et al. (2017)
Phenylalanine	–
Proline	Lynch et al. (2011), Prudente et al. (2017), Lineros et al. (2018)
Others	
Abscisic acid (ABA)	Kulus et al. (2018a, b)
Coconut water	Distabanjong et al. (2015)
Polyamines	Ramon et al. (2002)

The addition of these compounds, which have characteristics that allow greater interaction with macromolecules such as DNA, RNA, proteins and phospholipids, plays an active role in regulating the physical and chemical properties of membranes and in modulating enzymatic activities (Galston and Sawhney 1990; Pieruzzi et al. 2011). Additionally, these compounds protect the cells against reactive oxygen species (ROS) reactions capable of causing lipid peroxidation, a process responsible for altering membrane integrity by denaturing proteins and causing nucleic acid rupture (Baťková et al. 2008; Uchendu et al. 2010).

For *Chrysanthemum* 'Lady Orange' shoot tips, the composition of the culture medium used during preculture had a significant influence on the survival and regeneration potential of the plant material, with increased survival observed for explants grown in medium supplemented with 0.06 M sucrose for 7 days (Zalewska and Kulus 2013). Similar results were observed in the cryopreservation of encapsulated and precultured *Rabdosia rubescens* shoot tips in medium containing a high sucrose concentration (0.4 M) combined with 2.0 M glycerol, which produced a regeneration rate of 85% (Ai et al. 2012).

However, for some types of explants, preculture directly in a culture medium with high sucrose content may be toxic and cause tissue oxidation, leading to a very low explant survival rate (Feng et al. 2013). Thus, the success of a cryopreservation protocol may be associated with modifications made to specific steps of standard protocols, according to the requirements of each explant and/or species under study.

2.3 Encapsulation and Dehydration

After preculturing the explants, the next step is to encapsulate them for subsequent dehydration and cooling. Explant encapsulation facilitates tolerance to methods employing low-moisture content, which would be highly detrimental or lethal for nonencapsulated samples. Dehydration can be accomplished rapidly through physical dehydration by exposing the newly prepared capsules over a predetermined period to conditions necessary for the abrupt withdrawal of water content from the capsules. This method is called encapsulation-dehydration and was developed by Fabre and Dereuddre (1990) (Fig. 1a). Osmotic dehydration, which is called encapsulation-vitrification and was first used in 1995 by Matsumoto et al., is a slower method than the former procedure. This technique consists of immersing the capsules into one or more solutions that allow the cell to tolerate the physical and chemical changes related to cooling and reheating, extracting water molecules from the intracellular environment and replacing them with sugar and other vitrifying compounds (Fig. 1b).

Physical dehydration of the encapsulated material occurs continuously and can be achieved by exposing the material in an open container to continuous ventilation in a laminar flow chamber. Generally, the temperature and relative humidity of the environment are not controlled in this type of dehydration and may vary according to locality and climatic conditions, but the decrease in water content should be

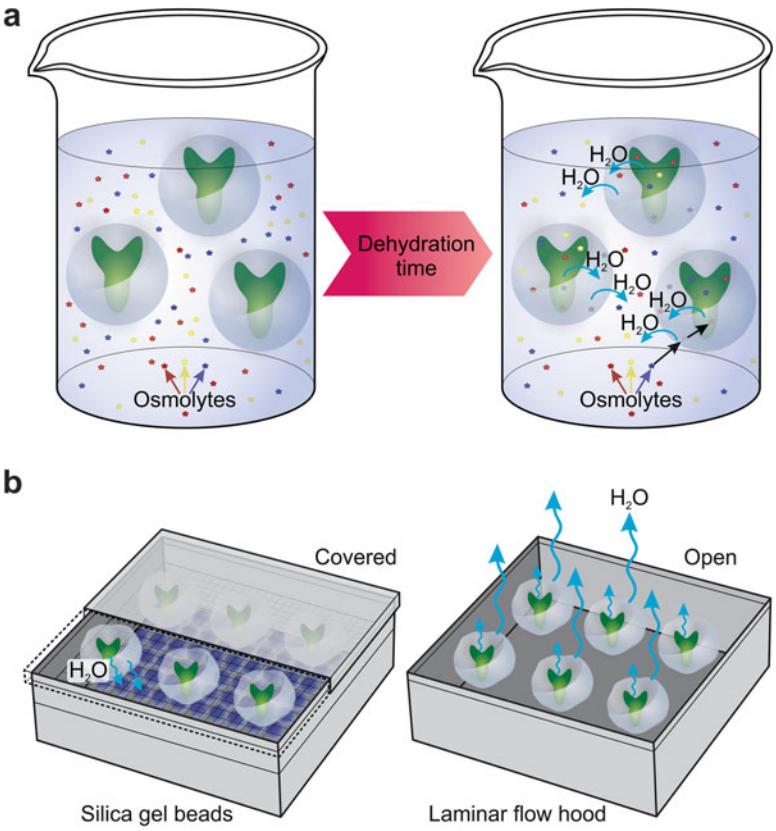

Fig. 1 Synthetic seed during osmotic dehydration (**a**) and physical dehydration with *silica gel beads* and inside a *laminar flow hood* (**b**)

controlled over the tested time for accuracy and repeatability of the protocol (Pammenter et al. 2002).

The use of hermetically sealed containers containing silica gel or saturated salt solutions is a relatively rapid method compared to laminar flow drying. The use of chambers containing saturated salt solutions has the advantage of controlling the relative humidity of the environment promoted by each salt at a given temperature, thus promoting a relative humidity balance between the material to be desiccated and the environment induced by the salt solution (Medeiros et al. 2006). Salts such as sodium chloride (75% relative humidity at 25 °C), sodium nitrite (64% relative humidity at 25 °C), calcium nitrate (52% relative humidity at 25 °C), magnesium (53% relative humidity at 25 °C), potassium hydroxide (8% relative humidity at 25 °C) and others are used in the preparation of saturated salt solutions for desiccation (Chen 2004).

Osmotic dehydration can be initiated by immersing the capsules in the loading solution, typically consisting of 0.4 M sucrose and 2.0 M glycerol, for 20 min at

room temperature, as a means of preparing the material for exposure to the vitrification solution. After exposure to the loading solution, the explants are immersed in cryoprotectants, such as plant vitrification solution 2 (PVS2: 3.26 M glycerol +2.42 M ethylene glycol +1.9 M dimethyl sulphoxide (DMSO) + 0.4 M sucrose at 0 °C) or plant vitrification solution 3 (PVS3: 50% glycerol +50% sucrose, at 0 °C), to promote the artificial vitrification of the cytoplasm and confer greater stability to the cell membranes during dehydration, avoiding injury due to chemical toxicity or excessive osmotic stress.

2.4 Cooling and Reheating

During rapid cooling, the dehydrated capsules may be directly immersed in liquid nitrogen or vitrification solution in cryotubes before immersion of the cryotubes in liquid nitrogen, reaching cooling rates of approximately −200 °C min^{-1} (Stanwood 1985).

Reheating must be performed quickly because when reheating material freshly removed from liquid nitrogen, temperatures between −15 °C and − 60 °C are considered critical, since they represent the range in which ice crystal nucleation and formation occur (Fowler and Toner 2006). The capsules may be immersed in a water bath at 37–40 °C for heating (heating rate of approximately 250 °C min^{-1}) for a given period of time.

2.5 Post-Thaw and Growth Recovery

Reestablishing growth after cryopreservation is a critical phase of the technique. At this point, the resumption of metabolic processes interrupted during capsule cooling should occur with minimal physical and osmotic disturbances.

Sample thawing significantly affects cell integrity, which may result in the degradation of cell wall-bound compounds (Tomaz et al. 2018). Thus, the composition of the postculture medium can be altered over the dwell time of the explants in this phase.

The addition of activated charcoal and antioxidants to the postculture medium within the first hours after thawing may reduce the osmotic stress caused by the transfer of the explants to a medium with low osmotic pressure. The aim is to eliminate the phenolic compounds produced by dead cells that intensify oxidation reactions (Thomas 2008). The culture conditions are also important, because studies have shown that incubating the plant material in a postculture medium in the dark for a short period of time can optimize the recovery and regeneration of the cryopreserved structures by decreasing the photo-oxidation of the material (Gonzalez-Arnao and Engelmann 2006).

After the critical period, the addition of growth regulators to the regeneration medium and transfer of the material to conditions with a photoperiod may stimulate cell proliferation and organ development (Prudente et al. 2017).

Previous studies have shown that cryopreservation alone did not cause the immediate death of protocorm-like bodies (PLBs) of the hybrid orchid Bratonia but triggered injuries that progressively worsened, resulting in the death of PLBs during postculture without the addition of stabilizing compounds (Popova et al. 2010).

Beads containing raspberry (*Rubus idaeus* L.) shoot tips were precultured in solid MS (Murashige and Skoog 1962) medium, and the cultures were kept in the dark at 22 ± 2 °C for 3 days and then transferred to the light conditions described for the standard culture. This condition was essential for the success of the encapsulation-vitrification and encapsulation-dehydration techniques applied to seven raspberry genotypes, which achieved mean survival and regeneration rates of 71% and 68%, respectively (Wang et al. 2005).

The cryopreservation of *Vitis* spp. shoot tips using the encapsulation-dehydration protocol resulted in regeneration values of 60% and 40% when the explants were postcultured in MS medium supplemented with 1 mg L^{-1} of 6-benzyladenine (BA) and 0.1 mg L^{-1} of 1-naphthaleneacetic acid (NAA).

These results reinforce the importance of the postculture conditions after reheating the samples to resume the growth of cryopreserved explants. It is evident that modifications to the different steps of the cryopreservation protocol are explant specific, in addition to being highly necessary to achieve the maximum survival and regeneration of the plant material (Fig. 2).

3 Other Uses of Synthetic Seed Cryopreservation

Cryopreservation may also be used for purposes other than germplasm conservation. Recently, cryopreservation was evaluated as a means of eliminating viruses from infected plants, a strategy called plant cryotherapy (Prudente et al. 2018).

In cryotherapy, the biological material is immersed in liquid nitrogen for a short period of time, usually 90 min (Cejas et al. 2012). Under these conditions, the infected cells are eliminated because their structures are weakened, with only the cells in the meristematic region being conserved (Wang and Valkonen 2009). The selected and uninfected cells remain alive due to the application of procedures that prevent water crystallization in the intracellular environment (Silva et al. 2013).

Considering the results of a decade of research, cryotherapy may be considered a rapid method capable of facilitating or even replacing some traditional methods used to eradicate plant microorganisms (Wang and Valkonen 2009). The main advantage of cryotherapy is the ability to treat a large number of samples simultaneously (Bhojwani and Dantu 2013), which confers speed to the method, decreases costs and increases the frequency of virus-free plants after regeneration (Wang and Valkonen 2009; Feng et al. 2013). Before beginning cryotherapy experiments, it is necessary to establish an adequate dehydration strategy for the plant material to reduce harm to the tissues, since the content of intracellular water is a critical factor for the efficiency of cryotherapy protocols (Engelmann 2011).

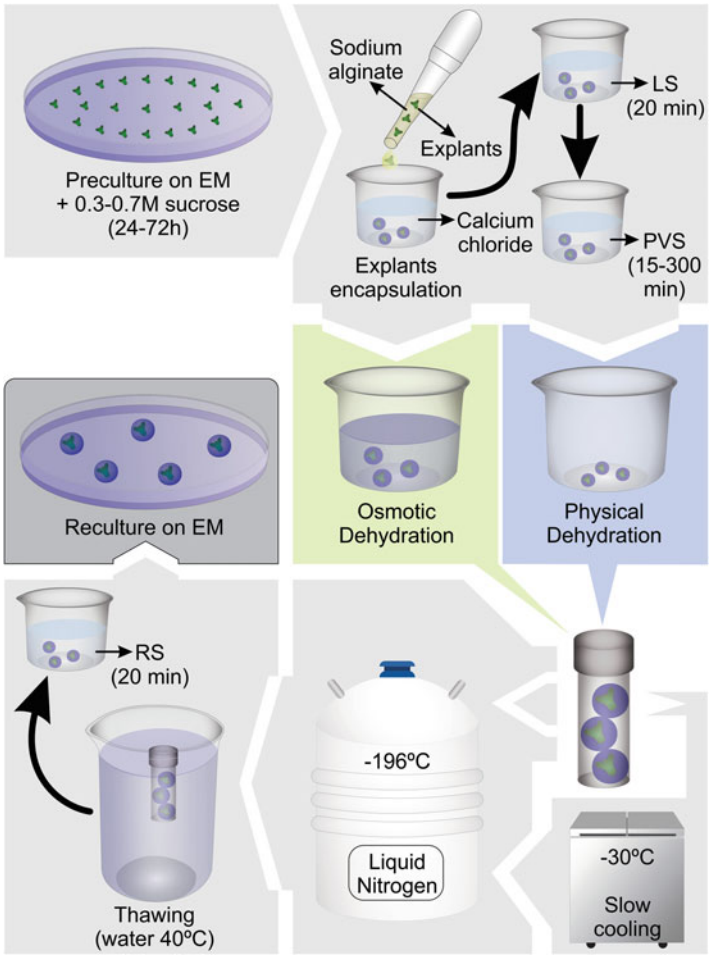

Fig. 2 General schematic representation of the complete protocol for the encapsulation-dehydration and encapsulation-vitrification techniques. *EM* establishment medium, *LS* loading solution, *PVS* plant vitrification solution, *RS* recovery solution, bold arrows = steps only required for encapsulation-vitrification; slow cooling is an alternative and optional method

Several cryopreservation techniques have been successfully applied for cryotherapy, including both encapsulation-dehydration (Wang et al. 2000, 2003; Bayati et al. 2011) and encapsulation-vitrification (Jeon et al. 2015) techniques.

4 Conclusion

Encapsulation-vitrification and encapsulation-dehydration protocols are highly efficient methods for the cryopreservation of a wide variety of species and explants sensitive to conventional cryopreservation methods.

Progress in the development and application of cryopreservation techniques will be made through a better understanding of the mechanisms involved in inducing explant tolerance to dehydration and cryopreservation after cooling. An equally important issue is the specific modifications made during each cryopreservation step, especially the addition of cryoprotective and osmoprotective compounds in the preculture phase, adequate dehydration of sensitive explants and improvements in the incubation conditions appropriate for the postculture phase and plant material regeneration.

5 Future Perspectives

The growing interest in improving cryopreservation techniques has led to an increase in the number of studies on the subject worldwide, making it possible, based on the current state of the art for some species, to store large amounts of delicate tissues using encapsulation, which has advantages such as the ease of handling explants. In the last 20 years, valuable studies have been published. Increasingly, the incorporation of new technologies has guaranteed the refinement of cryopreservation techniques and represents an advance for amplifying the genetic base of future varieties and for transporting and producing explants that may be regenerated at any time of the year.

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Progress and Challenges in the Application of Synthetic Seed Technology for Ex Situ Germplasm Conservation in Grapevine (*Vitis* spp.)

Angela Carra, Francesco Carimi, Jean Carlos Bettoni, and Ranjith Pathirana

Abstract Although grapevine (*Vitis* spp.) is one of the most ancient and important fruit crops, there is no concerted international effort to conserve its genetic resources, which are estimated to consist of 10–14,000 cultivars. Synthetic seed technology offers opportunities to conserve clonal genetic resources either in the form of quiescent somatic embryos or as encapsulated regenerable somatic tissue. Since the first report of somatic embryogenesis in grapevine in 1976, much research has been conducted into synchronising the process, maturation, dehydration, encapsulation and testing longevity under cold storage. Since the development of vitrification-based cryopreservation methods, both somatic embryos and other somatic tissue with meristematic regions have been used in cryopreservation experiments, and methods have been optimised to reach post-thaw regeneration percentages that satisfy gene bank standards for implementing cryopreservation. Nevertheless, improved protocols for 'difficult' genotypes are still needed for induction of somatic embryos and synchronising their formation, maturation and germination, as well as cryopreservation. As a result of these difficulties, conservation by cryopreservation has progressed using encapsulated shoot tips or axillary buds of tissue culture plants. Some vitrification-based methods use a droplet of vitrification solution to protect the shoot tips on an aluminium strip allowing faster freezing of tissue, an important factor for post-cryo-survival. The novel V cryo-plate method combines the advantages of both encapsulating the shoot tips in alginate beads that then adhere to the aluminium of the V cryo-plate, meaning manipulations

A. Carra · F. Carimi
Consiglio Nazionale delle Ricerche, Istituto di Bioscienze e BioRisorse, U.O.S. di Palermo, Palermo, Italy

J. C. Bettoni
Santa Catarina State University (UDESC), Lages, SC, Brazil

R. Pathirana (✉)
The New Zealand Institute for Plant and Food Research Limited, Palmerston North, New Zealand
e-mail: Ranjith.Pathirana@plantandfood.co.nz

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can be performed easily, and the high thermal conductivity of aluminium speeding processed of freezing and thawing. Cryopreservation of somatic embryos has been suggested as a way to conserve the diversity of wild *V. vinifera* ssp. *sylvestris*, and limited results obtained to date are promising.

Keywords Somatic embryogenesis · Non-zygotic embryo · Cryopreservation · Encapsulation · Vitrification · Embryo maturation · Tissue culture · Genetic resources

1 Introduction

Grapevine (*Vitis vinifera* L. ssp. *vinifera*) is a crop with great economic and cultural significance. There is 7.5 million ha under cultivation producing 75.8 million tons worldwide (OIV 2017). The genus *Vitis* belongs to the family Vitaceae and comprises about 60 inter-fertile species distributed through Europe, Asia and North America under subtropical, Mediterranean and continental-temperate climatic conditions (Carimi et al. 2011; Hancock 2004; Terral et al. 2010). While *V. vinifera* ssp. *vinifera* contributed almost entirely to the diversity of cultivars grown for fruit, juice and wine, other species such as the North American *V. rupestris*, *V. riparia, V. berlandieri or their hybrids* are used as rootstock for *V. vinifera* varieties, mainly due to their resistance to *Phylloxera* but also to other diseases such as *Oidium* and mildews, and for better tolerance to biotic stresses as well (Carimi et al. 2011; Terral et al. 2010). It is now established that grapevine as a crop originated independently in multiple regions, with evidence for West Asian and Caucasian centres of origin (Arroyo-Garcia et al. 2006; Imazio et al. 2013). It is also established that the cultivated form originated from the wild forms of *Vitis vinifera* L. ssp. *sylvestris* (Gmelin) Hegi (Bacilieri et al. 2013; Carimi et al. 2016; Myles et al. 2011).

Archaeobotanical and archaeological evidence for grape cultivation in the Caucasus region dates back to the sixth millennium BC (Imazio et al. 2013) and for the Aegean and Mesopotamian regions and in Egypt dates back to at least 4000 BC (Zohar and Horf 2000). Wild grapevine forms can be found from the South Atlantic coast of Europe through to the Western Himalayas and from sea level to 1000 m above sea level (Arnold et al. 1998). As grape cultivation spread to new areas, the cultivars probably hybridised with local wild and other cultivated genotypes resulting in selection of more adapted genotypes and cultivars with desirable fruit traits. Selection over several millennia has led to the development of an estimated 10,000–14,000 cultivars that are currently held in field collections (Alleweldt and Dettweiler 1994). Management of grapevine plants under field conditions is expensive and has resulted in loss of material in field gene banks (Maletić et al. 2008). Vegetative propagation adds another risk factor with potential for transfer of pathogens to new planting material. Therefore, development and application of plant tissue culture-based biotechnological approaches such as synthetic seeds and

cryopreservation are important for future conservation of *Vitis* germplasm. In this chapter, we present research on somatic embryogenesis in *Vitis* and its potential applications from an ex situ conservation perspective, with special reference to cryopreservation as a long-term conservation option.

2 Somatic Embryogenesis in Grapevine

Somatic embryogenesis (SEg) is the first step towards synthetic seed technology. The first successful induction of SEg was demonstrated in *V. vinifera* cv Cabernet sauvignon more than 40 years ago using unfertilised ovules (Mullins and Srinivasan 1976). Absence of a protective seed coat and lack of surrounding nutritive tissue make direct storage of somatic embryos (SE) difficult compared with zygotic embryos enclosed within seeds. Moreover, SE develop asynchronously and lack the quiescent resting stage of zygotic embryos (Gray et al. 1991). Therefore, research in synthetic seed technology has been directed not only at optimising induction of SE but also synchronising their induction (Jayasankar et al. 1999; Vasanth and Vivier 2011) and development, maturation (Vasanth and Vivier 2011), drying (McKersie et al. 1989; Senaratna et al. 1990; Madakadze and Senaratna 2000) and coating them to facilitate handling and supply of additional nutrients to the growing seedling (Senaratna 1992). Successful induction of SEg and maintenance of embryogenic cultures depend on a complex interaction between genotype, explant type used and culture conditions. Moreover, results are influenced by differential responses due to interaction of factors such as developmental stage of explants and nutrients and plant growth regulators (PGR) included in culture media (Carra et al. 2016; Prado et al. 2010b). Producing mature and well-developed somatic embryos that are able to grow into normal plants is challenging (Ji et al. 2017; Perrin et al. 2001).

2.1 Stages of Somatic Embryogenesis in Grapevine

SEg is the process by which somatic cells, under inductive conditions, generate embryogenic cells, which go through a series of morphological and biochemical changes that result in the formation of a SE. In grapevine, embryos pass through recognisable globular, heart, torpedo and early cotyledon stages, finally resulting in germinated embryos (Carimi et al. 2005).

Globular embryos usually appear on the surface of the embryogenic calli, and in this stage the young embryo is circular or slightly oblong and is still in close contact with the callus from which it was derived. Later, it detaches from the callus and the elongation of axial cells marks the beginning of tissue differentiation. At the end of the globular stage the two apical meristems are present and they persist through the heart-shaped to the torpedo stages. During the transition from the torpedo stage to the germinated embryo, grapevine somatic embryos undergo changes characteristic

of germination, e.g. radicle growth, tannin accumulation in the central cylinder and acquisition of an external suberin sheath (Faure et al. 1996; Ammirato 1987).

2.2 *Explant, Genotype and Growth Regulator Effects on Somatic Embryogenesis of Grapevine*

The influence of genotype, explant and PGR on somatic embryogenesis in grapevine including significant interactions between those factors have been demonstrated by several authors (Table 1).

Genotype is considered to be one of the most important factors affecting plant regeneration in vitro (Conde et al. 2008; Landi and Mezzetti 2006; Rodríguez et al. 2008). In grapevine, genotypes vary relatively widely in their embryogenic potential, and although several protocols have been published, methods still need improvement to optimise media and explant combinations to initiate embryogenic cultures from recalcitrant genotypes (Carra et al. 2016; Gambino et al. 2011a). SEg has been successfully achieved from different genotypes of *Vitis latifolia*, *V. longifolia*, *V. rupestris*, *V. rotundifolia, V. vinifera* L. ssp. *vinifera* and *V. vinifera* L. ssp. *sylvestris* starting from different explants (Carimi et al. 2013, 2016). Anthers have been widely used and embryogenic callus has been obtained for a remarkable number of genotypes (Martinelli and Gribaudo 2009). SEg has also been achieved from different reproductive organs like ovaries (López-Pérez et al. 2005), stigmas and styles (Carimi et al. 2005; Carra et al. 2016; Morgana et al. 2004), anthers (Gribaudo et al. 2004; Kikkert et al. 2005), anther filaments (Acanda et al. 2013; Perrin et al. 2004) and whole flowers (Gambino et al. 2007). Even if less common, SE can be induced from tissues derived from vegetative structures like tendrils (Salunkhe et al. 1997), leaf discs (Das et al. 2002), leaves and petioles (Martinelli et al. 1993) and stem nodal explants (Maillot et al. 2006, 2016).

The most common tissue culture media for inducing SE are based on MS (Murashige and Skoog 1962), NN (Nitsch and Nitsch 1969) or B5 (Gamborg et al. 1968) salts supplemented with different PGR. Usually auxins are the most important PGR to induce SE, and the auxin most frequently used is 2,4-dichlorophenoxyacetic acid (2,4-D). Indole-3-acetic acid (IAA), 2-naphthoxyacetic acid (NOA) and naphthalene acetic acid (NAA) are also used. When cytokinins are used in SEg induction media, they are added to the culture medium together with auxins. The most used cytokinin, 6-benzylaminopurine (BAP), supplemented at different concentrations depending on the type of explant and genotype, is used to initiate embryogenic cultures in combination with 2,4-D. Urea derivatives like thidiazuron (TDZ) or N-(2-chloro-4-pyridyl)-N-phenylurea (4-CPPU) in combination with auxins were effective when used in the induction phase in anther, pistil and ovary culture of *V. vinifera* (Acanda et al. 2013; Bouamama-Gzara et al. 2017; Carra et al. 2016; Kikkert et al. 2005). After embryogenic callus has been induced, in some cases development of somatic embryos is achieved by reducing or removing auxin from the culture medium (Coutos-Thevenot et al. 1992).

Table 1 Successful somatic embryogenesis protocols in *Vitis*

Explant	Species (cultivar/genotype)	PGR	References
Anther filaments	*Vitis vinifera* (Hencha)	2,4-D 9 μM + TDZ 10 μM	Bouamama-Gzara et al. (2017)
Anther filaments	Rootstock (6 genotypes); *V. vinifera* (13 cvs)	2,4-D 4.5 μM + BAP 9 μM	Perrin et al. (2004)
Anthers	*Vitis vinifera* (6 cvs)	NOA 5 μM + BAP 2 μM	Vasanth and Vivier (2011)
Anthers	*Vitis berlandieri* × *Vitis rupestris; V. vinifera* (2 cvs)	NOA 5 μM + BAP 1 μM	Ben-Amar et al. (2013)
Anthers	*V. berlandieri x V. rupestris* (110 Richter)	2,4-D + TDZ combinations	Forgács et al. (2017)
Anthers	*V. vinifera x V. rupestris* (Gloryvine and other cvs)	2,4-D 5 μM + BAP 1 μM	Rajasekaran and Mullins (1979)
Anthers	*V. rupestris, V. longii, V. vinifera* (Grenache)	2,4-D 5 μM + BAP 1 μM	Mullins and Rajasekaran (1980)
Anthers	*V. vinifera* (Cabernet Sauvignon)	2,4-D 4.5 μM + BAP 1.1 μM	Mauro et al. (1986)
Anthers	*V. riparia*	2,4-D 5 μM + BAP 0.9 μM	Mozsar and Sule (1994)
Anthers	*V. vinifera* (4 cvs)	2,4-D 9 μM + BAP 0.9 μM	Perl et al. (1995)
Anthers	*V. vinifera* (Grenache Noir)	2,4-D 4.5 μM + BAP 1.1 μM	Faure et al. (1996)
Anthers	*Vitis* ssp. (10 cvs)	2,4-D 5 μM + BAP 1 μM	Torregrosa (1998)
Anthers	*V. vinifera* (Sultana)	2,4-D 4.5 μM + BAP 9 μM	Franks et al. (1998)
Anthers	*V. latifolia*	2,4-D 20 μM + BAP 9 μM	Salunkhe et al. (1999)
Anthers	*V. vinifera* (15 cvs)	2,4-D 2.5 μM + BAP 0.8 μM	Perrin et al. (2001)
Anthers	*V. vinifera* (9 cvs)	2,4-D 9 μM + TDZ 11.35 μM	Bouamama et al. (2007)
Anthers	*V. vinifera* (Macabeo and Tempranillo)	2,4-D 5 μM + BAP 1 μM	Cutanda et al. (2008)
Anthers	*V. vinifera* (16 cvs); *Vitis* hybrids (11 cvs)	2,4-D 5 μM + TDZ 0.2 μM; 2,4-D 5 μM + BAP 0.4 μM; 2,4-D 2.5 μM + NOA 2.5 μM + 4-CPPU 5 μM	Oláh et al. (2009)
Anthers	*Vitis vinifera* (Pinot noir)	2,4-D 4.4 μM + BAP 4.4 μM	Larrouy et al. (2017)
Anthers and gynoecia	*Vitis vinifera* (Manicure Finger)	2,4-D 4.5 μM + BAP 4.4 μM	Xu et al. (2014)

(continued)

Table 1 (continued)

Explant	Species (cultivar/genotype)	PGR	References
Anthers and ovaries	*V. longii* (microsperma)	2,4-D 5 μM + BAP 1 μM	Gray and Mortensen (1987)
Anthers and ovaries	*V. vinifera* (2 cvs); *V. berlandieri* x *V. rupestris* (110 Richter); *V. berlandieri* x *V. riparia* (5BB)	2,4-D 9 μM + BAP 4.4 μM	Martinelli et al. (2001)
Anthers and ovaries	*V. vinifera* (6 cvs); *Vitis* hybrid (Chancellor); *V. labruscana* (Concord and Niagara)	2,4-D 2.5 μM + NOA 2.5 μM + 4-CPPU 5 μM	Kikkert et al. (2005)
Anthers and ovaries	*V. vinifera* (7 cvs)	2,4-D 4.5 μM + BAP 8.9 μM	Croce et al. (2005)
Anthers and ovaries	*V. vinifera* (Touriga Nacional)	2,4-D 4.5 μM + BAP 8.9 μM	Pinto-Sintra (2007)
Anthers and pistils	*Vitis vinifera* L. ssp. *sylvestris*	NOA 5 μM + BAP 4.4 μM	Carimi et al. (2016)
Anthers, ovaries and flower buds	*Vitis vinifera* (Chardonnay)	2,4-D + BAP + picloram several concentrations	Dai et al. (2015)
Filaments	*V. vinifera*; *V. labruscana* (Bailey)	2,4-D 1 μM + TDZ 1 μM	Nakajima and Matsuta (2003)
Floral explants	*V. vinifera* (Albariño)	2,4-D 4.52 μM + BAP 4.4 μM; 2,4-D 4.52 μM + NOA 2.5 μM+ 4-CPPU 5 μM	Saporta et al. (2014)
Immature anthers	*Vitis vinifera* (Mencía)	2,4-D 4.5 μM + BAP 9 μM	Prado et al. (2014)
Immature anthers and ovaries	*V. vinifera* (8 cvs)	2,4-D 4.52 μM + BAP 4.4 μM; 2,4-D 4.52 μM + NOA 2.5 μM+ 4-CPPU 5 μM	Vidal et al. (2009)
Immature anthers and ovaries	*V. vinifera* (6 cvs)	2,4-D 4.5-9 μM + BAP 4.5-9 μM	Prado et al. (2010a)
Immature leaves	*V. vinifera* (Thompson Seedless)	NOA 2.5 μM + BAP 5 μM+ 2,4-D 2.5 μM	Tapia et al. (2009)
Immature leaves and stamens	*V. rotundifolia* (2 cvs); *V. vinifera* (4 cvs)	2,4-D + BAP + NOA several combinations	Li et al. (2014)
Immature seeds	*Vitis vinifera* L (14 cvs)	TDZ 0.90 μM	San Pedro et al. (2017)
Leaves regenerated in vitro	*V. vinifera* (Crimson Seedless)	NOA 4.95 μM+ BAP 4.44 μM+ phenylalanine 5.0 mM	Nookaraju and Agrawal (2012)

(continued)

Table 1 (continued)

Explant	Species (cultivar/genotype)	PGR	References
Leaves regenerated in vitro	*Vitis vinifera* (Crimson Seedless)	NOA 5 μM+ BAP 4.5 μM + several amino acids	Nookaraju and Agrawal (2013)
Leaves regenerated in vitro	*V. vinifera* (Velika)	2,4-D 9 μM+ IAA 6 μM + BAP 4.4 μM + GA_3 1.8 μM	Tsvetkov et al. (2014)
Leaves	*V. vinifera* (Koshusanjaku)	2,4-D 5-10 μM + TDZ or 4-CPPU 5-10 μM	Matsuta and Hirabayashi (1989)
Leaves	*Vitis* hybrids (Seyval Blanc and Chancellor); *V. thunbergii*	NOA 20 μM + BAP 40 μM or TDZ 4 μM	Harst (1995)
Leaves	*V. rupestris* (du Lot)	2,4-D 9 μM + BAP 9 μM	Tsolova and Atanassov (1996)
Leaves	*V. vinifera* (Podarok Magaracha)	2,4-D 9 μM + BAP 4.4 μM then NAA 5.4 μM + BAP 4.4 μM	Kuksova et al. (1997)
Leaves	*V. vinifera* (Pusa Seedless, Beauty Seedless, Perlette and Nashik)	2,4-D 0.45 μM + BAP 4.5 μM	Das et al. (2002)
Leaves and anthers	*V. vinifera*; *V. rupestris* (several cvs)	NOA 5 μM + BAP 0.9–4.5 μM	Stamp and Meredith (1988b)
Leaves and petioles	*V. rupestris*	IAA 5.7 μM or IBA 0.5 μM	Martinelli et al. (1993)
Leaves and petioles	*V. rotundifolia* (Regale and Fry)	2,4-D 9 μM + BAP 4.4 μM, then NAA 10.7 μM + BAP 0.9 μM	Robacker (1993)
Ovaries	*V. labruscana* (Fredonia and Niagara)	2,4-D 9 μM + BAP 1 μM + IASP 17 μM then 2,4-D 2 μM or 2,4-D 2 μM + IASP 4 μM	Motoike et al. (2001)
Ovaries, anther filaments, stigmas and styles	*Vitis vinifera* (8 cvs)	2,4-D 5 μM + CPPU 5 μM; NOA 20 μM + TDZ 4 μM; NOA 5 μM + BAP 4.4 μM; NOA 10 μM + BAP 4.4 μM	Carra et al. (2016)
Ovules	*V. vinifera* (Cabernet Sauvignon and Grenache), *Vitis* hybrid (Gloryvine)	2,4-D 5 μM + BAP 1 μM	Srinivasan and Mullins (1980)
Ovules	*V. labruscana* (Kyoto)	2,4-D 1 μM + TDZ 0.2 μM	Nakajima et al. (2000)
PEM from anthers and ovaries	*V. vinifera* (Chardonnay)	2,4-D 2 μM	Jayasankar et al. (2001)
Petioles	*V. vinifera*; *Vitis* hybrid	BAP 2.2 μM	Zlenko et al. (2002)

(continued)

Table 1 (continued)

Explant	Species (cultivar/genotype)	PGR	References
Protoplasts	*Vitis* hybrids (Seyval Blanc)	NOA 20 μM + TDZ 4 μM	Reustle et al. (1995)
Protoplasts	*V. vinifera* (Koshusanjaku)	NAA 10.7 μM + BAP 2.2 μM	Zhu et al. (1997)
Seed integuments	*V. vinifera* (Autumn Royal Seedless)	2,4-D 9 μM + BAP 4 μM	Xu and Lu (2009)
Stamen filaments	*Vitis vinifera* (Mencía)	2,4-D 1 μM+ TDZ 4.5 μM	Acanda et al. (2013)
Stamen filaments	*Vitis vinifera* (Mencía)	2,4-D 1 μM+ TDZ 4.5 μM	Acanda et al. (2015)
Stamens	*V. vinifera* L. (Thompson Seedless)	2,4-D 2.25 μM + BAP 18 μM	Zhou et al. (2014)
Stamens and pistils	*Vitis vinifera* L. (4 cvs)	2,4-D 4.5 μM + BAP 8.9 μM	Gambino et al. (2011b)
Stem segments with a unique axillary bud	*Vitis vinifera* (Chardonnay)	2,4-D + BAP at several concentrations	Maillot et al. (2016)
Styles and stigmas	*V. vinifera* (Sugraone)	NOA 5 μM + BAP 9 μM	Morgana et al. (2004)
Styles and stigmas	*V. vinifera* (4 cvs)	NOA 9.9 μM+ BAP 4.5 μM; BAP 9 μM	Carimi et al. (2005)
Tender stems	*V. amurensis* Rupr	NAA 0.5 μM + BAP 2.2 μM	Sun et al. (2016)
Tendrils	*V. vinifera* (3 cvs)	NAA 0.4 μM + BAP 10 μM + GA_3 2.8 μM	Salunkhe et al. (1997)
Unopened leaves, petioles and fully opened leaves	*Vitis rotundifolia* (5 cvs)	2,4-D 9 μM + BAP 4.4 μM	Dhekney et al. (2011)
Whole flower bud	*Vitis vinifera* (Thompson Seedless)	NOA 2.5 μM + 2,4-D 2.3 μM + 4-CPPU 4 μM	Ji et al. (2017)
Whole flowers, anthers and ovaries	*V. vinifera* (8 cvs); *V. berlandieri x V. rupestris* (110R)	2,4-D 4.5 μM + BAP 9 μM	Martinelli et al. (2003)
Whole flowers, anthers and ovaries	*V. vinifera* (8 cvs); *V. berlandieri x V. rupestris* (110R)	2,4-D 4.5 μM + BAP 9 μM	Gribaudo et al. (2004)
Whole flowers, anthers and ovaries	*V. vinifera* (8 cvs); *V. berlandieri x V. rupestris* (110R)	2,4-D 4.5 μM + BAP 9 μM	Gambino et al. (2007)
Whole flowers, anthers and ovaries	*V. vinifera* (8 cvs); *V. berlandieri x V. rupestris* (110R)	2,4-D 4.5 μM + BAP 9 μM	Cadavid-Labrada et al. (2008)
Zygotic embryos	*V. vinifera* (4 cvs); *V. longii*	NOA 5 μM + BAP 0.9 μM	Stamp and Meredith (1988a)

(continued)

Table 1 (continued)

Explant	Species (cultivar/genotype)	PGR	References
Zygotic embryos	*V. rotundifolia* (5 cvs)	NOA 5 μM + BAP 0.9 μM	Gray (1992)

2,4-D 2,4-dichlorophenoxyacetic acid, *BAP* 6-benzyladenine, *4-CPPU* N-(2-chloro-4-pyridyl)-N-′-phenylurea, *cvs* cultivars, *IAA* indole-3-acetic acid, *IBA* indole-3-butyric acid, *IASP* indole-3-acetyl-L-aspartic acid, *NAA* 2-naphthaleneacetic acid, *NOA* 2-naphthoxyacetic acid, *PEM* proembryogenic masses, *PGR* plant growth regulators, *TDZ* N-(1,2,3-thiadiazol-5-yl)-N-′-phenylurea (thidiazuron)

Physical culture conditions significantly influence the embryogenic response. By manipulating light intensity and temperature, Das et al. (2002) developed an efficient leaf-disc method for the regeneration of plants via SEg. Other workers incubate cultures in total darkness until embryogenic callus is formed and then transfer cultures to the light (Oláh et al. 2009; Prado et al. 2010a). Several parameters such as composition of basal medium, medium pH, type of gelling agent, presence of activated charcoal, carbohydrate source and light intensity and spectral composition can influence culture success (Jittayasothorn et al. 2007). Type of culture, liquid or solid, influences the final result, and the liquid culture is to be preferred in the induction phase while structured embryogenic callus grows better on solid media (Jayasankar et al. 2003; Mullins and Srinivasan 1976). Moreover, quality of SE affects regeneration frequency, which varies depending on type of culture: SE cultured on solid media often show dormancy, whereas in liquid media SE were not dormant and showed higher regeneration efficiency (Jayasankar et al. 2003; Mullins and Srinivasan 1976).

SEg is significantly influenced by the developmental stage of explants, and preconditioning treatments have been proved to be crucial in determining the final result. Gribaudo et al. (2004) screened six different developmental stages of anthers to initiate SEg cultures and identified a correlation between anther stage and SEg efficiency. The optimal developmental stage is related to genotype, and for *V. vinifera* 'Chardonnay' and 'Barbera', higher rates of SEg had been obtained when explants were collected at early stages, while in the rootstock '110R', later stages proved to be more efficient. In *V. lambruscana*, the best results were obtained with anthers collected 20 days before anthesis (Nakajima et al. 2000), while for eight Tunisian cultivars of *V. vinifera*, the best performance was achieved with anthers at the tetrad stage of microspore development (Bouamama et al. 2007). Similar results were reported more recently also for *V. vinifera* 'Sultana', 'Red Globe' and 'Merlot' (Vasanth and Vivier 2017).

2.3 *Synchronisation of Somatic Embryo Production and Their Germination*

The synchronisation of SE development is a critical step for taking advantage of SEg for applications, such as micropropagation, germplasm conservation and genetic

transformation, and for gene expression studies. Anatomical and developmental studies towards this goal have been made, comparing SE development in solid media with those in suspension cultures (Jayasankar et al. 2003). Developing SEs on solid media had large cotyledons, little or no visible suspensor structure and a relatively undeveloped concave shoot apical meristem, whereas those developing in liquid media had smaller cotyledons, a distinct suspensor and a flat-to-convex shoot apical meristem. Also, SEs derived from solid media exhibited physiological dormancy and did not germinate without a dormancy-breaking treatment (Jayasankar et al. 2003). Faure et al. (1996) observed asynchronous development of SEs when embryogenic callus cultured in liquid media supplemented with 2,4-D and BAP was transferred to the same liquid media devoid of PGR. They described developmental stages of proembryos in PGR supplemented media, followed by development of globular stage embryos in PGR-free media (Faure et al. 1996).

Jayasankar et al. (1999) reported a high degree of synchronisation of somatic embryo production by alternating solid and liquid media for culture. However, further development of somatic embryos was better achieved in semi-solid media. Embryo germination was influenced by genotype and culture conditions. SEs derived from suspension cultures of 'Chardonnay' did not have a dormant phase and germinated precociously, whereas 'Thompson Seedless' SEs did not develop beyond the heart stage in liquid medium (Jayasankar et al. 1999). In contrast, Zlenko et al. (2002, 2005) successfully converted somatic embryos developed on liquid induction media by subculturing them on liquid media supplemented with BAP and GA_3, or GA_3 alone. Plant regeneration appears to be easier for SEs of Muscadine grapes (*Muscadinia rotundifolia*), as Lu et al. (2007) were able to germinate more than 95% of synchronously produced SEs using suspension cultures established in woody plant medium (Lloyd and McCown 1980). In contrast, in some cultivars of *V. vinifera* such as 'Grenache noir', the germination of somatic embryos is very poor due to their inability to utilise the starch and lipids accumulated in cotyledons at the torpedo stage (Faure and Aarrouf 1994). Another problem with suspension cultures of grapevine is the browning of the suspension of cells and medium, due to production of phenolic compounds. Jayasankar et al. (1999) overcame this problem by sieving the larger cell masses, which contained differentiated somatic embryos. After three subcultures, they managed to produce large numbers of cytoplasm-rich proembryonic masses (PEM). Two cultivars used behaved quite differently: 'Chardonnay' produced SEs from PEMs directly upon subculture onto hormone-free media, whereas 'Thompson Seedless' did not advance beyond the heart stage. The use of conditioned medium has been reported to facilitate embryo proliferation and conversion. Supplementing liquid culture media with arabinogalactan-proteins has been shown to facilitate cell proliferation of grapevine embryogenic cultures (Ben Amar et al. 2007). Recently, an improved protocol based on the dynamic maintenance of culture medium has been proposed. Forgács et al. (2017) stated that culture density affects both the amount of differentiating embryos and their stage of development in 'Richter 110'. Results show that to achieve full synchrony, it is essential to use low cell density obtained through readjusting it to the initial value every week.

2.4 Applications of Somatic Embryogenesis in Grapevine

SEg is the preferred method for cell to plant regeneration in *V. vinifera* L. and has been reported for several important *Vitis* species. SEg has been widely applied in crop genetic improvement and it was suggested as a specific tool to induce somaclonal variation in grapevine and specifically to amplify clonal variability (Acanda et al. 2015; Desperrier et al. 2003; Kuksova et al. 1997; Torregrosa et al. 2001). SEs have proven to be an excellent resource for mutations (Pathirana 2011) even if this approach does not guarantee a good rate of SE induction and conversion into plantlets, which is strictly genotype dependent. For this reason, mutagenesis has not been extensively used for grapevine improvement even if physical and chemical mutagens have been investigated. Recently, production of colchicine- and oryzalin-induced polyploid mutants has been described starting from SEs of 'Crimson seedless', 'BRS Clara' and 'Mencia' (Acanda et al. 2015; Sinski et al. 2014). Currently, SEg is the most suitable tool for in vitro manipulation of the *Vitis* genus (Kikkert et al. 2001; Martinelli and Mandolino 2001). For this reason, mutagenesis of somatic embryos has become an interesting tool in genomics programs to assign gene function, particularly since the availability of the draft genome sequence of grapevine (Jaillon et al. 2007; Velasco et al. 2007).

Virus infections cause severe economic losses in grapevine with several viruses known to negatively influence grape quality and yield. SEg has been proved to be highly effective in eliminating some viruses, either alone or in combination with thermotherapy (Gambino et al. 2006, 2009). Bouamama-Gzara et al. (2017) reported 100% elimination of *Grapevine leafroll-associated virus 3*, *Grapevine stem pitting-associated virus* and *Grapevine virus A* from the Tunisian cultivar Hencha through SEg.

Other applications include the isolation of natural somatic mutants (Boss and Thomas 2002; Franks et al. 2002), and germplasm conservation through synthetic seeds technology and cryopreservation (Brambilla 1999; Carimi et al. 2016; Gray and Compton 1993; Gray et al. 1991; Jayasankar et al. 2005).

SEs have been tested as a resource for conservation of grapevine germplasm. Jayasankar et al. (2005) cultured mature somatic embryos at low density (250 embryos/40 ml liquid media) for 2 weeks, dried them in the laminar flow for ~4 h and sealed in Petri dishes. Ninety percent of these dehydrated SEs produced plants after 42 months of storage at 4 °C in *V. vinifera* 'Chardonnay'. *V. vinifera* 'Autumn Seedless' SEs were generally poor in germination from the beginning but did not show any deterioration during the month storage trial, rather their conversion rate increased from 30 to 40% over the storage period, a statistically significant difference (Jayasankar et al. 2005).

Possible use of SEs for clonal propagation was demonstrated by Jayasankar et al. (2001) using *V. vinifera* 'Chardonnay'. They blot-dried mature SEs and germinated directly in different sterilised agar-free potting media under aseptic conditions. Commercial potting mixture overlaid by sand produced the best results with 32% of the SEs growing into normal plantlets ready for greenhouse acclimation. They proposed encapsulating SEs for further improvements to the propagation methodology.

2.5 *Genetic Stability of Somatic Embryo-Derived Grapevine Plants*

Plantlets derived from an in vitro culture may exhibit somaclonal variations (Larkin and Scowcroft 1981). There are two types of somaclonal variations: heritable and epigenetic (Skirvin et al. 1994). Heritable changes in in vitro cultures occur at higher frequency than occurs spontaneously in seeds or grafted plants (Prado et al. 2010b; Sahijram et al. 2003). Therefore, somaclonal variations may constitute a serious problem in clonal propagation systems aimed at the preservation of plant genetic integrity (Sahijram et al. 2003). However, somaclonal variations may also be exploited as a source of new genetic variability for crop improvement, especially in trees and long-lived perennial species and vegetatively propagated plants (Karp 1995). Methods for detecting somaclonal variation were extensively reviewed by Bairu et al. (2011). Somaclonal variants can be detected using various techniques that are broadly categorised as morphological and molecular detection techniques.

Morphological variants can be easily detected based on characters such as differences in plant stature, leaf morphology or pigmentation abnormalities (Israeli et al. 1991). In grapevine, variation in leaf shape was reported for several somaclones of 'Grenache' (Martinez et al. 1997). Variability in cropping level, berry weight and vigour are also reported (Torregrosa et al. 2011). DNA-based techniques, such as restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), microsatellite or inter-simple sequence repeat (ISSR) and amplified fragment length polymorphism (AFLP) markers, are valuable tools for analysing the genetic fidelity of in vitro propagated plants. Variations in tissue culture-derived plants can also be generated by changes in methylation (Schellenbaum et al. 2008) and ploidy (Martinelli and Gribaudo 2001). Genetic profiles determined by DNA markers (ISSR, AFLP, RAPD, SSR) have shown that genetic fidelity is not compromised during SE, except in very rare instances (Prado et al. 2010b; Yang et al. 2006). Flow cytometry has been used in *V. vinifera* to verify the ploidy level and ploidy stability of SE-derived plants (Leal et al. 2006; Prado et al. 2010b). Researchers reported a low percentage of somaclonal variation, taking into account that all the embryos were produced from callus tissue, which is prone to genetic variability (Sato et al. 2011; Smulders and De Klerk 2011).

3 Conservation of High-Health Germplasm Through Cryopreservation

Plant cryopreservation is the storage of structurally intact cells, tissues or organs in liquid nitrogen (LN) or its vapour phase at ultra-low temperatures, mainly for conservation of genetic resources. The temperature in LN (−196 °C) or its vapour phase ensures the cellular activity is slowed to the point of cessation where vital functions such as enzymatic activity, gene function and respiration cease, thus

arresting the cell aging. Hence in theory, cryopreserved cells cannot age beyond the physiological point at which they were placed in cryo storage (Benson 2008). Thus, cryopreservation provides a means to conserve plant genetic resources in gene banks that are otherwise under threat when maintained in the field, particularly in the case of clonally propagated species such as grapevine that are expensive to duplicate in different locations or to maintain as in vitro cultures.

3.1 Developing High-Health Plants for Industry and Conservation

In addition to storage of genetic resources for future use, cryopreservation has been used in recent years to eradicate infecting microorganisms in many horticultural species including grapevine (Bettoni et al. 2016; Wang and Valkonen 2007, 2009; Bi et al. 2018a; Wang et al. 2003). Table 2 gives details of the grapevine cultivars from which different viruses were eradicated using cryopreservation techniques.

There are a number of advantages of using cryotherapy to obtain high-health plants for the industry compared with traditional methods of virus eradication. Among these advantages, the ability to treat a large number of samples and genotypes simultaneously, higher frequency of virus-free plants and cost-effectiveness are the major benefits of cryo-based technologies (Bettoni et al. 2016).

4 Methods Used for Grapevine Cryopreservation

Although the first method of cryopreservation of plant material developed using winter dormant twigs of *Salix*, mulberry and poplar (Sakai 1960) may apply to frost hardy grapevine, its wider application may be limited. Further manipulation of freezing tolerance, for example, through exogenous abscisic acid (Rubio et al. 2018), may enable the use of the method in grapevine. Currently, the dormant bud method is successfully used mainly in apple for long-term preservation (Höfer 2015; Pathirana et al. 2018; Towill and Bonnart 2005). On the other hand, development of vitrification-based methods applied to embryogenic masses, SEs, shoot tips and axillary buds from tissue cultured plants has progressed rapidly.

Vitrification is freezing of a solution without crystallisation. The water molecules are sparsely distributed in highly concentrated solutions, including cytoplasm of highly dehydrated plant tissue, particularly in the non-vacuolar, highly cytoplasmic meristematic tissue. Snap freezing of such tissue in LN will result in the solution transitioning to a vitreous (glassy) state. In this state, the metabolic activity in cells ceases and cells survive without aging, theoretically for eternity.

The vitrification method of cryopreservation involves dehydration of cells/tissues in glycerol-based cryoprotective solutions such as plant vitrification solution

Table 2 Efficiency of plant regeneration and virus eradication in cryopreserved grapevine

Grapevine cultivar	Method of cryopreservation	Efficiency (% explants regenerated)	Virus	Efficiency of eradication (% clean)	References
Bruti	Vitrification	50	*Grapevine virus A*	97	Wang et al. (2003)
Bruti	Encapsulation-dehydration	62	*Grapevine virus A*	97	Wang et al. (2003)
Black	Encapsulation-dehydration	59	*Grapevine virus A*	42	Bayati et al. (2011)
Chardonnay	Droplet-vitrification	30.7	*Grapevine fanleaf virus*	77.8	Markovic et al. (2015)
Cabernet sauvignon	Droplet-vitrification	41.6	*GLRaV-3*[a]	100	Markovic et al. (2015)
Chardonnay	Droplet-vitrification	13.0	*GLRaV-3*	100	Pathirana et al. (2015)
Pinot gris	Droplet-vitrification	13.6	*GLRaV-2*[b]	100	Pathirana et al. (2015)
Sauvignon blanc 316	Droplet-vitrification	15.7	*GLRaV-2*	100	Pathirana et al. (2015)
Lakemont seedless	Droplet-vitrification	16.2	*GLRaV-3*	100	Pathirana et al. (2015)
Sauvignon blanc	Droplet-vitrification	30.0	*GLRaV-1*[c] and *GLRaV-3*	100 (both viruses)	Pathirana et al. (2015)
Cabernet sauvignon	Droplet-vitrification	23–59[c]	*GLRaV-3*	100	Bi et al. (2018a)
Chardonnay	Droplet-vitrification	47	*GLRaV-3*	100	Bi et al. (2018a)
Kyoho (*V. vinifera* x *V. labrusca*)	Droplet-vitrification	51	*GLRaV-3*	100	Bi et al. (2018a)
Hunan-1 (*V. pseudoreticulata*)	Droplet-vitrification	43	*GLRaV-3*	100	Bi et al. (2018a)

[a]*Grapevine leafroll-associated virus 3*
[b]*Grapevine leafroll-associated virus 2*
[c]*Grapevine leafroll-associated virus 1*

2 (PVS2) (Sakai et al. 1990). Penetrative cryoprotectants such as ethylene glycol and dimethyl sulphoxide (DMSO) in vitrification solutions are believed to prevent ice formation by interfering with hydrogen bonding between water molecules (Best 2015), and they also displace water within the cytoplasm, thus supporting dehydration of tissue.

There are several methods of vitrification described for plant tissue:

(a) Two-Step Freezing

This older method was mainly used for 'unprotected' (Ezawa et al. 1989) and protected shoot tips (Plessis et al. 1991, 1993) and axillary buds through encapsulation in alginate beads (Miaja et al. 2000; Zhao et al. 2001), as well as for embryogenic cell suspensions (Dussert et al. 1991, 1992; Ben-Amar et al. 2013). Ezawa et al. (1989) used a freezing solution containing 10% DMSO and 60 g/L sucrose to cryopreserve *Vitis labrusca* shoot tips (1–2 mm). After holding shoot tips for 2 h in this solution at room temperature, they cooled the shoot tips in the same solution at a rate of −0.5 °C per minute using a programmable freezer and found that freezing to −30 °C followed by immersion in LN gave better regeneration than those frozen to −20 °C or − 40 °C and immersed in LN. Also, shoot tips from twigs harvested from the field in November and December from Hokkaido Research Station fields in Japan responded better to freezing in LN than those harvested in October (Ezawa et al. 1989).

Zhao et al. (2001) encapsulated axillary buds of four *V. vinifera* accessions and treated them in increasing sucrose concentrations from 0.1 to 1 M and desiccated with silica gel to 21% moisture content then slowly cooled to −40 °C at a rate of −0.2 °C/min before immersion in LN. They found that plants maintained without subculture for 3 or 4 months and those that received a cold-acclimation of 1 month at 5 °C were amenable to cryopreservation. Shoot tips from younger plants in tissue culture did not regenerate after cryopreservation by this method.

Dussert et al. (1991) used anthers of rootstock '41B' (*V. vinifera* 'Chasselas' x *V. berlandieri*) to induce embryogenic callus from which they produced embryogenic cell cultures. These were used in their cryopreservation experiments. They demonstrated that fast freezing is not effective for cryopreservation of embryogenic cell suspensions and only two-step freezing allowed successful cryopreservation. DMSO (5% w/v) and 0.25 M maltose in the freezing solution gave the highest (>60%) regeneration rates after slow cooling to −40 °C at the rate of −0.5 °C/min followed by immersion in LN. Another important step is the incubation of the cells in the medium for 1 h at 0 °C as a pretreatment step before slow freezing. The optimum post-thaw culture medium consisted of a semi-solid medium supplemented with 2 mg/L naphthoxyacetic acid (Dussert et al. 1992). Activated charcoal (0.1%) helped prevent browning of the cells after thawing, but regrowth was reduced in its presence. A minimum of 6 days in the semi-solid medium was essential for recovery of cells after cryopreservation (Dussert et al. 1992).

Ben-Amar et al. (2013) compared a two-step freezing method with direct freezing for cryopreservation of embryogenic cell lines of three grapevine accessions: Rootstock 110 Richter (*V. berlandieri* x *V. rupestris*) and *V. vinifera* cv. Riesling and

cv. Tempranillo. For both procedures, they employed pre-culture of embryogenic cell masses in increasing sucrose concentrations from 0.25, 0.5, 0.75 M through to 1 M over 4 days either before encapsulating in alginate beads (dehydration-encapsulation) or after (encapsulation-dehydration). Then, the cells in beads were treated for 3 days in a mixture of 2 M glycerol and 0.4 M sucrose before either being dehydrated in the laminar flow hood airflow before direct immersion in LN or maintained at 0 °C for 30 min, for 45 min at −20 °C or for 45 min at −80 °C before transfer to LN. Direct freezing recorded significantly higher regeneration rates (43.3–78%) than slow freezing (15.3–25.3%) in all three accessions. However, the slow freezing method employed was different from that of previous workers who demonstrated better results when a programmed freezer was used (Dussert et al. 1991, 1992; Zhao et al. 2001) to control the rate of temperature drop.

The method used by Plessis et al. (1993) and Miaja et al. (2000) involved sucrose pre-culture of shoot tips in alginate beads followed by dehydration for 4 h in the laminar hood airflow that resulted in 30% moisture content (Miaja et al. 2000), followed by slow freezing at 0.5 °C/min to −80 °C and transfer to LN. While Plessis et al. (1993) recorded 24% shoot tip survival in *V. vinifera* 'Chardonnay' by this two-step encapsulation-dehydration procedure, Miaja et al. (2000) did not recover whole plants in three cultivars ('Nebbiolo', 'Barbera' and 'Brachetto'), although cells of some explants showed viability when tested using fluorescent microscopy.

In the three-step vitrification method, explants are stepwise pre-cultured in tissue culture media enriched with increasing sucrose concentrations over several days followed by osmoprotection using a glycerol and sucrose mixture and finally PVS2 dehydration. In this method, after the final treatment in vitrification solution, explants are held in a cryotube with vitrification solution and transferred directly to LN. Researchers describe such vitrification methods as two-step or three-step vitrification methods; however, this should not be confused with two-step freezing method where the explants are first cooled slowly at a selected rate and then transferred to LN after reaching a designated temperature such as −30 °C.

(b) Encapsulation-Dehydration

This method was first reported by Fabre and Dereuddre (1990) for cryopreservation of *Solanum* shoot tips and is based on the technology developed for the production of artificial seeds. Encapsulation-dehydration has been experimented with in well over 70 different plant species (Engelmann et al. 2008). Plessis et al. (1991) were the first to report this method to cryopreserve grapevine shoot tips. In this method, explants are placed into alginate solution (3% Na-alginate (w/v), 2 M glycerol and 0.4 M sucrose in MS liquid medium with no $CaCl_2$), and then individual explants in alginate solution are transferred by pipette to a calcium chloride solution (0.1 M calcium chloride, 2 M glycerol and 0.4 M sucrose in MS liquid medium) in a droplet. The explant gets entrapped in beads by ionotropic gelation, with $CaCl_2$ acting as the crosslinking agent. Crosslinking is complete in about 30 min and produces beads of 4–5 mm diameter containing an explant. Then the beads are pre-cultured on basal MS media supplemented with increasing sucrose concentrations of 0.25, 0.5, 0.75 and 1 M for 4 days, a step per day, before partial desiccation in the air current of a

laminar flow hood or on silica gel to desiccate the beads to about 16 to 20% moisture content (Markovic et al. 2013; Wang et al. 2000). The dehydration period can vary depending on the ambient temperature and humidity, especially for air-drying in a laminar flow hood. The two desiccation methods produce similar results with respect to *Vitis* shoot regrowth as demonstrated in rootstock LN33 (Couderc 1613 × *Vitis vinifera* 'Thompson Seedless'). The highest survival (60%) was achieved when beads were dehydrated for 7 h in laminar flow hood airflow or for 4.5 h on silica gel, with a final bead moisture content of about 16% (Wang et al. 2000). However, desiccation on silica gel is easier to reproduce than air-drying, since the room conditions are variable among labs or even at different periods of the year within the same lab. After desiccation, the beads are transferred to cryovials (~10 beads per cryovial) and plunged into LN. For testing viability, cryovials are warmed in a 40 °C water bath for 3 min and encapsulated shoot tips are cultured on recovery medium. Encapsulation-dehydration procedures for different *Vitis* genotypes have been tested using both direct immersion in LN after osmoprotection (Bayati et al. 2011; Bi et al. 2018a; Carimi et al. 2016; Dussert et al. 1991; Wang et al. 2000, 2002, 2004) and by two-step freezing (Dussert et al. 1991, 1992; Ezawa et al. 1989; Miaja et al. 2000, 2004; Plessis et al. 1991, 1993; Zhao et al. 2001) focusing on several *Vitis* species.

(c) Encapsulation-Vitrification

This method is a combination of encapsulation-dehydration and vitrification procedures, where explants are encapsulated in alginate beads and dehydrated chemically using vitrification solutions; it combines the advantages of ease of manipulation of encapsulated explants and the fast dehydration by vitrification (Matsumoto and Niino 2017; Sakai and Engelmann 2007). The encapsulation-vitrification method has been studied less than other cryopreservation procedures for grapevine (Bettoni et al. 2016). In the study of Benelli et al. (2003), shoot tips from rootstock Kober 5BB (*Vitis berlandieri* x *Vitis riparia*) were excised from cold acclimated cultures (3-week at 4 °C) and encapsulated in 3% calcium alginate. The beads were placed in cryovials and exposed to PVS2 at 0 °C for 90 min. Cryovials containing shoot tips and PVS2 were then immersed in LN and stored. For recovery, LN-stored cryovials were warmed in a 40 °C water bath and encapsulated shoot tips were cultured on recovery medium. This protocol resulted in low regrowth levels, not specified by the authors (Benelli et al. 2003).

(d) Droplet-Vitrification

This technique is a variant of vitrification-based cryopreservation and was derived from the droplet-freezing technique developed by Kartha et al. (1982) for freezing cassava shoot tips using slow cooling and then modified by Schafermenuhr et al. (1994) for potato shoot tip cryopreservation. In this method, explants are osmoprotected, exposed to vitrification solution, placed onto individual droplets or a thin layer of PVS2 on aluminium foil strips and then transferred to LN (Bi et al. 2018b; Pathirana et al. 2016). The main advantage of this technique over the traditional vitrification procedures is the possibility of achieving faster freezing and warming rates due to the direct contact of explants with LN (Panis et al. 2011; Sakai and Engelmann 2007). Aluminium, with its high thermal conductivity, further

facilitates fast freezing of the shoot tips. Droplet-vitrification has been applied successfully to in vitro grown shoot tips of diverse plant species, including grapes (Bi et al. 2018b; Carimi et al. 2016; Hassan and Haggag 2013; Markovic et al. 2013; Pathirana et al. 2016; Volk et al. 2018). So far, droplet-vitrification appears to be a promising method to overcome species- and genotype-specific responses that have been bottlenecks for the widespread use of *Vitis* cryopreservation (Bi et al. 2018b; Volk et al. 2018). Recently, improvements in droplet-vitrification protocols for cryopreservation of *Vitis* have been reported, and these are associated with improving the explant quality (Markovic et al. 2013) and pretreatment conditions, adding antioxidants (Bi et al. 2018a) and elicitors of defence proteins such as salicylic acid (Bi et al. 2018b; Pathirana et al. 2016; Volk et al. 2018). Volk et al. (2018) reported a widely applicable *Vitis* droplet-vitrification method and applied it to nine species. In their protocol, shoot tips were excised from nodal sections that were grown on MS medium containing 0.2 mg L^{-1} N^6-benzyladenine, 1 mM salicylic acid, 1 mM glutathione (reduced form) and 1 mM ascorbic acid for 2 weeks. Then, the shoot tips were pre-cultured on half-strength MS medium containing 0.3 M sucrose, 0.1 mM salicylic acid, 1 mM ascorbic acid and 1 mM glutathione (reduced form) for 3 days, treated with loading solution (half-strength MS + 2 M glycerol +0.4 M sucrose) for 20 min and then with half-strength PVS2 for 30 min at 22 °C followed by full-strength PVS2 treatment for 90 min at 0 °C prior to immersion in LN. Shoot tips were warmed in unloading solution (half-strength MS + 1.2 M sucrose) for 20 min and post-thaw cultured for shoot regrowth. This cryo-protocol resulted in 24–43% shoot regrowth and averaged 35 ± 2% across nine *Vitis* species. Bettoni et al. (2018) reported a similar *Vitis* droplet-vitrification method to that described by Volk et al. (2018); in addition, they showed the possibility of cryopreserving *Vitis vinifera* 'Chardonnay' shoot tips without first introducing the accession into tissue culture, using plants from a growth chamber. Nodal sections were harvested from the growth chamber plants, surface sterilised and plated on pretreatment medium for 2 weeks, and then shoot tips (1 mm) were dissected and pre-cultured for 3 days. The pretreatment and pre-culture medium were those described above by Volk et al. (2018), with addition of the plant preservation mixture (PPM®, 1.5% v/v) to reduce microbial contamination. Pre-cultured shoot tips were treated with loading solution for 20 min, followed by half-strength PVS2 for 30 min at 22 °C and then full-strength PVS2 treatment for 30 min at 0 °C prior to immersion in LN. Following LN, the shoot tips were warmed in unloading solution for 20 min and post-thaw cultured for shoot regrowth. About 43% shoot regrowth was obtained for *V. vinifera* 'Chardonnay'.

(e) V Cryo-plate Method

The recently developed V cryo-plate technique is an optimisation of the encapsulation and droplet-vitrification methods and combines advantages of both (Yamamoto et al. 2011). Explant handling is facilitated by droplet encapsulated plant tissues adhering to the wells of aluminium cryo-plates, and at the same time, this process aids fast cooling and warming rates, an important requirement for successful cryopreservation protocols (Niino et al. 2013; Panis et al. 2005; Yamamoto et al. 2011).

The main advantages of the cryo-plate method, over the other vitrification-based cryopreservation techniques, are simplicity and user-friendliness; samples can be easily transferred between solutions with minimal risk of mechanical injuries because all treatments can be carried out on cryo-plates with shoot tips attached, i.e. the cryo-plate is manipulated only after the shoot tips have adhered onto it (Rafique et al. 2015). The V cryo-plate method has been successfully applied to a diverse range of species (Matsumoto and Niino 2017). Bettoni et al. (2019) reported a practical and promising cryopreservation protocol for in vitro grapes shoot tips using the V cryo-plate method. In this protocol, microcuttings were grown on MS pretreatment medium supplemented with 0.2 mg L^{-1} N^6-benzyladenine, 1 mM salicylic acid, 1 mM glutathione (reduced form) and 1 mM ascorbic acid for 2 weeks, and then shoot tips (1 mm) were dissected from the shoots and pre-cultured on half-strength MS medium containing 0.3 M sucrose, 0.1 mM salicylic acid, 1 mM ascorbic acid and 1 mM glutathione (reduced form) for 3 days. Pre-cultured shoot tips were attached to wells of a cryo-plate with alginate (2% Na-alginate (w/v)) and calcium (0.1 M calcium chloride) beads and treated with loading solution for 30 min and then exposed to PVS2 at 22 °C for 40 min. The cryo-plate was then immersed in LN for 1 h and warmed into unloading solution for 20 min, and the alginate beads were transferred onto the recovery medium. This protocol resulted in 68–70% shoot regrowth in *Vitis* accessions *V. aestivalis* (DVIT 1408) and *Vitis jacquemontii* (PI 135726).

5 Conclusions and Future Perspectives

Since the first report of SEg in grapevine, much research has been conducted on improving the conditions for inducing embryogenic cultures and on manipulating those to obtain mature SEs synchronously. Lack of synchrony and consistency in SE formation, poor maturation, lack of a quiescent stage resulting in autonomous germination and inability to dehydrate them unlike zygotic embryos are problems. There are only a few reports of attempts to store dehydrated SEs of grapevine, but some accessions have stored well up to 2 years at 4 °C. Variability in response in different genotypes requires optimising media and conditions for recalcitrant genotypes. As a result of these difficulties, conservation by cryopreservation has progressed using encapsulated shoot tips or axillary buds of tissue culture plants. One vitrification-based method uses a droplet of vitrification solution to protect the shoot tips on an aluminium strip allowing faster freezing of tissue, an important factor for post-cryo-survival. The novel V cryo-plate method combines the advantage of both encapsulation in alginate beads to adhere the explants, so manipulations can be performed easily, and the high thermal conductivity of aluminium. Cryopreservation of SEs has been suggested as a way to conserve the diversity of wild *V. vinifera* ssp. *sylvestris*, and the limited results are promising. Further research towards incorporating dehydration tolerance in SEs would allow further progress in using SEs as the clonal unit of conservation.

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Cryopreservation of Grapevine Shoot Tips from In Vitro Plants Using Droplet Vitrification and V Cryo-plate Techniques

Jean Carlos Bettoni, Ranjith Pathirana, Remi Bonnart, Ashley Shepherd, and Gayle Volk

Abstract The availability of and easy access to *Vitis* genetic resources are essential for future breeding program advances. Cryopreservation is currently considered an ideal means for the long-term preservation of clonally propagated plant genetic resources. When robust methods for cryopreservation of *Vitis* spp. are available, there is an opportunity to preserve collections for extended lengths of time with minimal cost and labor requirements and a low risk of loss. This chapter describes the droplet vitrification and V cryo-plate protocols that have been shown to be effective for the cryopreservation of multiple *V. vinifera* genotypes and other *Vitis* species.

Keywords *Vitis* · Tissue culture · Conservation · Genetic resources · Germplasm

1 Introduction

Grapevine is among the most economically important fruit crops cultivated in all continents with high cultural significance in many countries. Grapevine has a rich genetic diversity, with most of the globally important cultivars belonging to *Vitis vinifera* L. subsp. *vinifera.* Other *Vitis* species, as well as *Muscadinia rotundifolia*, are important to provide valuable genes for breeding new elite cultivars and rootstocks through traditional and biotechnological approaches (Carimi et al. 2011; Eibach et al. 2009).

J. C. Bettoni (✉)
Oak Ridge Institute for Science and Education (ORISE), Oak Ridge, TN, USA

USDA-ARS National Laboratory for Genetic Resources Preservation, Fort Collins, CO, USA

R. Pathirana (✉)
The New Zealand Institute for Plant & Food Research Limited, Palmerston North, New Zealand
e-mail: Ranjith.Pathirana@plantandfood.co.nz

R. Bonnart · A. Shepherd · G. Volk
USDA-ARS National Laboratory for Genetic Resources Preservation, Fort Collins, CO, USA

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Availability and easy access to *Vitis* genetic resources are essential for future breeding program advances (Alleweldt and Dettweiler 1994; Carimi et al. 2016; Wang et al. 2018).

Cryopreservation is a valuable technique for the safe, long-term conservation of *Vitis* genetic resources that complements traditional field collections. *Vitis* genetic resources are vulnerable to pathogens and environmental disasters in field genebanks, which can lead to the depletion of germplasm that may be needed for future breeding efforts (Bi et al. 2017; Markovic et al. 2013; Pathirana et al. 2016).

Cryopreservation is the storage of biological samples in liquid nitrogen (LN, −196 °C) or liquid nitrogen vapor (LNV, −165 to −196 °C). Cryopreservation methods have been used to securely conserve plant genebank collections for the long term. In LN or LNV, viable explants are preserved in a state where cellular division and metabolic processes are minimized, preserving the structure and function of the biological system (Benelli et al. 2013; Benson 2008; Wang et al. 2014). Materials are kept in a state that minimizes genetic drift. It is also cost-effective to maintain collections for extended periods of time in LN compared to field or in vitro collections (Benson 2008; Reed 2014).

Several cryopreservation protocols have been described for grapes (Bettoni et al. 2016; Bi et al. 2017, 2018a; Pathirana et al. 2016). Among these, droplet vitrification has been demonstrated as the most effective across diverse *Vitis* species (Bettoni et al. 2019a, b; Bi et al. 2018b; Volk et al. 2018). Droplet vitrification makes use of ultra-fast shoot tip cooling and warming rates, an important requirement for successful cryopreservation protocols based on vitrification (Benson and Harding 2012). Recently, a novel cryopreservation protocol based on the V cryo-plate using Plant Vitrification Solution 2 (PVS2) (Sakai et al. 1990) has been developed (Yamamoto et al. 2011). This method simplifies handling of explants at different stages of cryopreservation by vitrification maintaining the fast freezing and thawing rates of droplet vitrification, over 4000 °C min^{-1} of treated explants (Yamamoto et al. 2011). The V cryo-plate method was successfully applied to a diverse range of genetic resources, including grapevine (Bettoni et al. 2019c).

Access to reliable cryopreservation methods is important for the widespread application of cryopreservation technologies for *Vitis* genetic resources (Reed et al. 2004). The aim of this chapter is to share the most successful protocols for cryopreserving grapevine, based on droplet vitrification and V cryo-plate techniques. The described protocols were effective for cryopreserving multiple *Vitis* species and genotypes.

2 Materials

2.1 Tissue Culture Facilities and General Equipment

1. Precision balance.
2. Magnetic stirrer.
3. pH meter.
4. Autoclave.
5. Laminar airflow cabinet.

6. Glass bead sterilizer.
7. Stereomicroscope.
8. Refrigerator to store prepared media and solutions.
9. Culture room with controlled temperature (25 °C) and photoperiod (16 h).
10. Forceps, scalpels and scalpel blades, pruning shears.
11. Glass pipettes.
12. Sterile Petri dishes (∅ 90 mm).
13. Volumetric flasks.
14. Sterile plastic Petri dishes (100 × 25 mm; 60 × 15 mm).
15. Sterile glass containers (jars).
16. Plastic wrap.
17. Sterile aluminum cryo-plate n° 2 (37 × 7 × 0.5 mm) (Taiyo Nippon Sanso Corp., Tokyo, Japan).
18. Sterile aluminum foil strips (~6 × 25 mm) (can be sterilized in glass Petri plates).
19. Micropipette.
20. LN Dewar for cryopreservation (600 mL).
21. Sterile filter paper strips.
22. Cryotubes.
23. Long-term LN storage tank.

2.2 Culture Media and Solutions

Prepare all the media and solutions using distilled H_2O and reagents with high analytical purity grade, and store at 4 °C, except for vitamins that should be stored at −20 °C. Murashige and Skoog (1962) (MS) salts formulation can be found in Table 1. MS medium can also be purchased from suppliers as powder mixture with all salts or without ammonium.

2.2.1 Stock Solutions

Plant Growth Regulators All plant growth regulators (PGR) are prepared at a concentration of 0.1 mg mL^{-1}. Dissolve the quantity of PGR in a small volume of 5 M NaOH and distilled H_2O to the desired volume, and store the PGR stock solutions at 4 °C.

Salicylic Acid Salicylic acid stock solution is prepared at a concentration of 0.1 mg mL^{-1}. Dissolve the quantity of salicylic acid in a small volume of 95% ethanol and distilled H_2O to the desired volume and store at 4 °C.

Vitamins The stock solution of MS medium vitamins is prepared according to the formulations given in Table 1. *Vitis* vitamins used in recovery media are composed of 100 mg L^{-1} myo-inositol, 10 mg L^{-1} thiamine HCl, 1 mg L^{-1} nicotinic acid, 1 mg L^{-1}

Table 1 Composition of the Murashige and Skoog (1962) culture medium

Macroelements	**mg L^{-1}**
NH_4NO_3	1650
$CaCl_2 \cdot 2H_2O$	440
$MgSO_4 \cdot 7H_2O$	370
KH_2PO_4	170
KNO_3	1900
Microelements	**mg L^{-1}**
$MnSO_4 \cdot H_2O$	16.9
$ZnSO_4 \cdot 7H_2O$	8.60
H_3BO_3	6.20
KI	0.83
$Na_2MoO_4 \cdot 2H_2O$	0.25
$CuSO_4 \cdot 5H_2O$	0.025
$CoCl_2 \cdot 6H_2O$	0.025
FeEDTA	**mg L^{-1}**
$FeSO_4 \cdot 7H_2O$	27.8
$Na_2EDTA \cdot 2H_2O$	37.3
Vitamins	**g L^{-1}**
Glycine	2.0
Nicotinic acid	0.5
Pyridoxine-HCl	0.5
Thiamine-HCl	0.1
Myo-inositol	100.0
Sucrose	30,000
pH	5.7–5.8

pyridoxine HCl, 1 mg L^{-1} calcium pantothenate, 0.01 mg L^{-1} biotin, and 2 mg L^{-1} glycine (Volk et al. 2018). Dispense both vitamins into aliquots, and store at −20 °C.

2.2.2 Shoot Initiation and Maintenance Medium

MS medium containing 30 g L^{-1} sucrose, 1.14 μM indole-3-acetic acid (IAA), and 2.5 g L^{-1} gellan gum at pH 5.7 (pH 6.4 prior to autoclaving) (*see* **Note 1**).

2.2.3 Pretreatment Medium

MS medium containing 30 g L^{-1} sucrose, 0.89 μM benzyladenine (BA), 0.1 mM salicylic acid, 1 mM ascorbic acid, 1 mM glutathione (reduced form), and 3 g L^{-1} gellan gum at pH 5.7 (pH 6.8 prior to autoclaving) (*see* **Note 2**).

2.2.4 Pre-culture Medium

Half-strength MS medium containing 0.3 M sucrose, 0.1 mM salicylic acid, 1 mM ascorbic acid, 1 mM glutathione (reduced form), and 8 g L^{-1} agar at pH 5.7 (pH 6.8 prior to autoclaving) (*see* **Note 2**).

2.2.5 Calcium Chloride Solution

MS medium containing 0.1 M calcium chloride and 0.4 M sucrose at pH 5.7 (pH 6.4 prior to autoclaving).

2.2.6 Sodium Alginate Solution

Calcium-free MS medium supplemented with 0.4 M sucrose and 2% (w/v) sodium alginate (medium viscosity – 3500 cps; Sigma® A-2033) at pH 5.7 (pH 6.4 prior to autoclaving).

2.2.7 Loading Solution

Half-strength MS medium containing 2 M glycerol and 0.4 M sucrose at pH 5.7 (pH 6.9 prior to autoclaving).

2.2.8 Half-Strength PVS2 Solution

Half-strength MS medium containing 15% (w/v) glycerol, 7.5% (w/v) ethylene glycol (EG), 7.5% (w/v) dimethyl sulfoxide (DMSO), and 0.4 M sucrose at pH 5.8 (*see* **Note 3**).

2.2.9 Full-Strength PVS2 Solution

Half-strength MS medium containing 30% (w/v) glycerol, 15% (w/v) EG, 15% (w/v) DMSO, and 0.4 M sucrose at pH 5.8 (*see* **Note 3**).

2.2.10 Unloading Solution

Half-strength MS medium containing 1.2 M sucrose at pH 5.7 (pH 7.55 prior to autoclaving).

2.2.11 Recovery Medium 1

Half-strength MS macroelements without ammonium (NH_4), MS microelements, and *Vitis* vitamins supplemented with 0.6 M sucrose and 8 g L^{-1} agar at pH 5.7 (pH 7.0 prior to autoclaving) (*see* **Note 2**).

2.2.12 Recovery Medium 2

Half-strength MS macroelements without NH_4, MS microelements, and *Vitis* vitamins supplemented with 30 g L^{-1} sucrose, 0.89 μM BA, and 8 g L^{-1} agar at pH 5.7 (pH 6.5 prior to autoclaving) (*see* **Note 2**).

2.2.13 Recovery Medium 3

Half-strength MS macroelements, MS microelements, and *Vitis* vitamins supplemented with 30 g L^{-1} sucrose, 0.89 μM BA, and 8 g L^{-1} agar at pH 5.7 (pH 6.5 prior to autoclaving) (*see* **Note 2**).

3 Methods

The droplet vitrification and V cryo-plate procedures for cryopreserving in vitro grapevine shoot tips given here were described by Volk et al. (2018) and Bettoni et al. (2019c), respectively. In vitro plants must first be established using source plant material from the field, greenhouse, or growth chamber. Once established, in vitro stock plants serve as the source of nodal sections, from which shoot tips are excised. The resulting shoot tips are then used for cryopreservation. An overview of the steps involved in these two protocols is shown in Fig. 1.

3.1 Collection and Surface Sterilization of Explants

Materials

1. Source plant material.
2. Isopropanol 70% (v/v).
3. Sterile distilled water.
4. Sodium hypochlorite (NaOCl) with 5% active Cl.
5. Tween 20®.
6. Erlenmeyer flask (150 mL).
7. Dissecting scissors.
8. Laminar airflow hood.

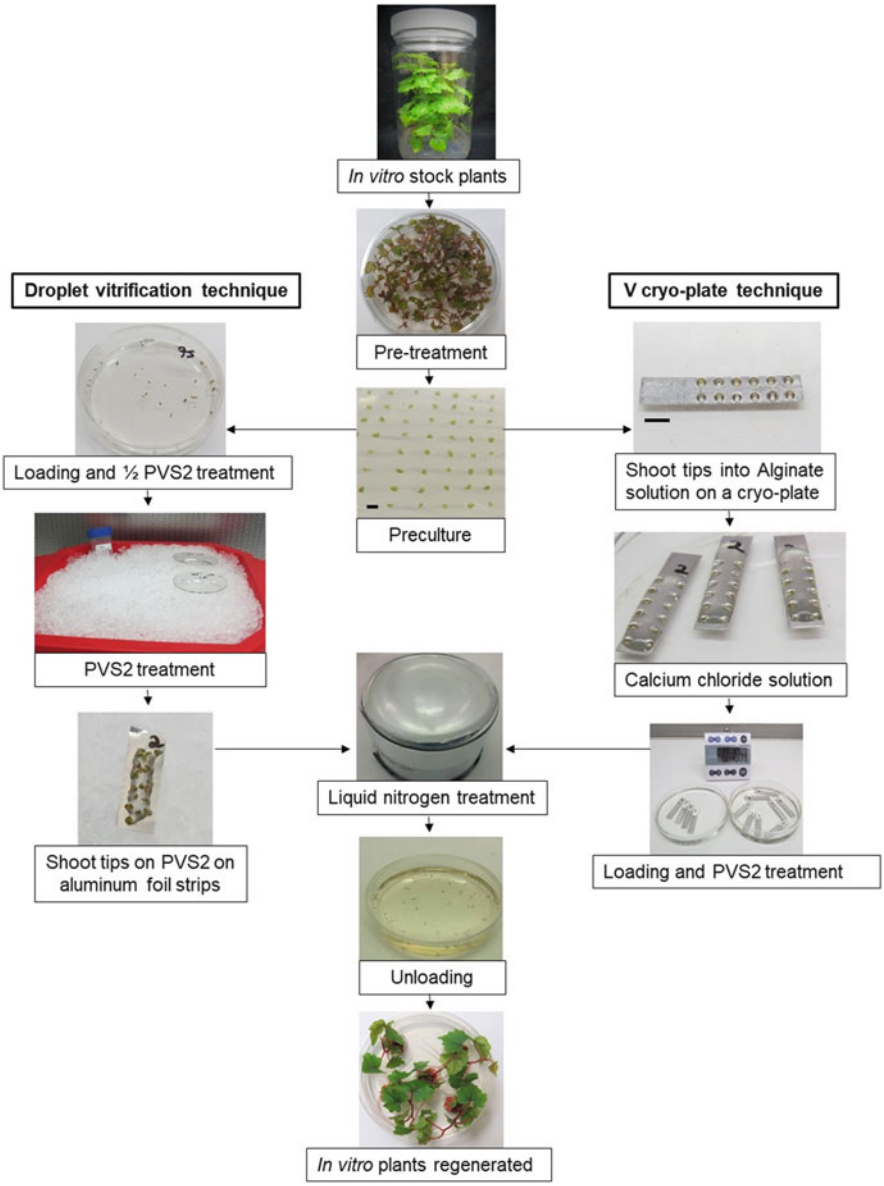

Fig. 1 Overview of the droplet vitrification (left) and V cryo-plate (right) procedures for cryopreservation of in vitro grapevine shoot tips

Methods

1. Select healthy source material without insects or diseases, preferably from plants maintained under optimal conditions in a growth chamber, greenhouse, or fresh flushes from the field.
2. Nodal sections approximately 2 cm long containing a single bud are harvested from plants, and all the leaves are removed.
3. Place cut shoots into clean 150 mL Erlenmeyer flask containing distilled water with three drops of Tween 20® (v/v).
4. In a laminar flow hood, drain water out of the Erlenmeyer flask, and sterilize nodal sections with 70% isopropanol for 1 min; then rinse twice for 1 min with distilled water. Treat nodal sections with 5% sodium hypochlorite and 0.1% Tween 20® (v/v) for 5 min, and rinse three times in sterile distilled water (*see* **Note 4**).

3.2 Establishment and Maintenance of In Vitro Stock Cultures

Materials

1. Tissue culture facilities and tools (laminar airflow cabinet, scalpel, forceps, tool sterilizer, sterile Petri dishes (∅ 90 mm), plastic wrap, culture room).
2. Glass test tubes (16 × 100 mm).
3. Glass culture vessels (89 × 170 mm).
4. Shoot initiation and maintenance medium.

Methods

1. Undamaged explants are placed into glass test tubes containing 10 mL of shoot initiation medium. Autoclave any tubes with media that appear cloudy or contaminated.
2. Place cultures in a growth room at 25 °C, under a photoperiod of 16 h light/8 h dark day^{-1} with a photosynthetic flux density of 20–50 μM m^{-2} s^{-1}.
3. Grow in vitro stock cultures in glass test tubes for 4 weeks, and then transfer new shoots to glass culture vessels (89 × 170 mm) containing 180 mL of shoot maintenance medium. Subculture every 8–12 weeks.

3.3 Pretreatment and Pre-culture of Shoot Tips for Cryopreservation

Materials

1. Tissue culture facilities and tools (laminar airflow cabinet, scalpel, forceps, tool sterilizer, sterile Petri dishes (∅ 90 mm), plastic wrap, stereomicroscope, culture room).
2. Plastic Petri dishes (100 × 25 mm, 60 × 15 mm).
3. Pretreatment and pre-culture medium.

Methods

1. Cut nodal sections from 2- to 3-month-old in vitro stock plants, and place nodal sections on 50 mL pretreatment medium in plastic Petri dishes (100 × 25 mm) at a density of 40 nodal sections per plate.
2. Maintain cultures under the same conditions as the in vitro stock cultures for 2–3 weeks until shoots are 1–2 cm in length (*see* **Note 5**).
3. Excise shoot tips (1 mm) from nodal sections. Place excised shoot tips on pre-culture medium (plastic Petri dishes 60 × 15 mm with 12 mL medium each one) for 3 days at 25 °C in the dark (*see* **Note 6**).

3.4 Cryopreservation

3.4.1 Droplet Vitrification Cryopreservation Method

Materials

1. Tissue culture facilities and tools (laminar airflow cabinet, forceps, tool sterilizer, pipette).
2. Plastic Petri dishes (60 × 15 mm).
3. Sterile aluminum foil strips (~6 × 25 mm).
4. Loading solution.
5. Half-strength and full-strength PVS2 solution.
6. LN Dewar for cryopreservation (600 mL).
7. LN.
8. Sterile cryotubes.
9. Long-term LN storage tank.

Methods

1. Remove dark-cultured shoot tips from pre-culture medium, and transfer to loading solution in plastic Petri dishes for 20 min at room temperature.
2. Remove loading solution from plastic Petri dishes using a sterile 2 mL transfer pipette, and add half-strength PVS2 to the shoot tips. Incubate at room temperature for 30 min.
3. Remove half-strength PVS2 from plastic Petri dishes using a sterile 2 mL transfer pipette, and add full-strength PVS2 at 0 °C for 90 min (*see* **Note 7**).
4. Two minutes before the end of PVS2 treatment, transfer shoot tips onto a thin layer of PVS2 (1 μL per explant) on sterile aluminum foil strips at 0 °C.
5. Plunge foil strips containing the shoot tips into LN in a 600 mL open-top container, and then place foil strips into pre-frozen sterile cryotubes.
6. For long-term storage, cryotubes containing foil strips with shoot tips are capped and stored in LN or LNV in an LN storage tank (*see* Sect. 3.6).

3.4.2 V Cryo-plate Cryopreservation Method

Materials

1. Tissue culture facilities and tools (laminar airflow cabinet, forceps, tool sterilizer, micropipette).
2. Sterile Petri dishes (∅ 90 mm).
3. Sterile aluminum cryo-plates No 2 (37 × 7 × 0.5 mm).
4. Calcium chloride solution; sodium alginate solution; loading solution.
5. Full-strength PVS2 solution.
6. Sterile filter paper.
7. LN Dewar for cryopreservation (600 mL).
8. LN.
9. Sterile cryotubes.
10. Long-term LN storage tank.

Methods

1. Set the cryo-plates on the Petri dish.
2. Place droplets of the sodium alginate solution into each cryo-plate well (2 μL per well), and place the pre-cultured shoot tips into each well (1 shoot tip per well). Add 1.5 μL sodium alginate solution to cover the shoot tips completely.
3. Add the calcium chloride solution dropwise to each cryo-plate until all the wells are covered. Polymerize for 20 min at room temperature (*see* **Note 8**).
4. Remove excess calcium chloride solution from the cryo-plate with a micropipette, and place the cryo-plate with attached shoot tips in a Petri dish containing loading solution. Incubate in loading solution at room temperature for 30 min (*see* **Note 9**).
5. Remove cryo-plate from loading solution, and use sterile filter paper to remove excess solution before the PVS2 treatment. Place the cryo-plate in a Petri dish with full-strength PVS2 at 22 °C for 40 min.
6. Remove cryo-plate from PVS2, and use sterile filter paper to remove excess PVS2. Transfer cryo-plate with shoot tips into LN in a 600 mL open-top container, and then insert cryo-plate with shoot tips into pre-frozen sterile cryotubes.
7. For long-term storage, cryotubes containing cryo-plates with shoot tips are capped and stored in LN or LNV in an LN storage tank (*see* Sect. 3.6).

3.5 Warming and Culturing of Cryopreserved Shoot Tips

Materials

1. Tissue culture facilities and tools (laminar airflow cabinet, forceps, tool sterilizer, pipette).
2. Sterile Petri dishes (∅ 90 mm).

3. Unloading solution.
4. Recovery medium 1, 2, and 3.

Methods

1. The aluminum foil strips or cryo-plates should be removed from the cryotube and warmed by inverting the foil strips or cryo-plates containing the shoot tips into 25 mL unloading solution in Petri dishes at 22 °C for 20 min (*see* **Note 10**).
2. Place the shoot tips onto recovery medium 1, and maintain overnight at 25 °C in the dark (*see* **Note 11**).
3. Transfer the shoot tips to recovery medium 2, and culture for 2 weeks at 25 °C in the dark (*see* **Note 11**).
4. Transfer the shoot tips to recovery medium 3, and maintain in the culture room under conditions described for stock cultures.

3.6 Determining the Number of Shoot Tips to be Processed in Cryobanks

In cryobanks, the number of samples processed per accession varies widely and is dependent upon technical staff availability, material availability, security level desired, etc. Additional cryovials of shoot tips should be processed to assess viability immediately after LN exposure to ensure the cryoprotective treatments were effective. Probabilistic tools have been developed to assist in determining the predicted number of shoot tips that should be cryopreserved, based on the number of explants processed initially and regrowth level after LN exposure (Dussert et al. 2003; Volk et al. 2016).

4 Notes

1. In an appropriate beaker, add distilled water up to half the final medium volume, add macro and micro salts of MS medium, with MS vitamins from the formulations in Table 1, or use MS powder mixture according to manufacturer's instruction. Add sucrose and plant growth regulator(s) (stir well after each addition). Bring to the desired volume with distilled H_2O, add gelling agent, mix well, and adjust the pH. Use test tubes for shoot initiation with 8–10 mL medium in each and glass culture vessels (89 × 170 mm) for the maintenance medium with 180 mL of medium in each one. Autoclave at 121 °C with time dependent upon the culture vessels size and medium volume. Store the medium at 4 °C, and use the culture medium within 1 month after its preparation.
2. After culture medium preparation, transfer it to an Erlenmeyer flask and seal; then sterilize by autoclaving at 121 °C with time related to the culture vessel size and volume of medium. In a laminar airflow hood, distribute the culture medium

into plastic Petri dishes, and then let plates cool with lids partially open for 30 min to evaporate excess moisture before storing. Store the medium at 4 °C, and use the culture medium within 1 month after preparation.

3. After pH adjustment, take the solution to the laminar flow hood, and carry out ultrafiltration using a filter pore diameter smaller than 0.22 μm. Sterilized PVS2 is stored at 4 °C. Use the solution within 1 month after its preparation.
4. To reduce the risk of contamination, this step should be performed in a laminar airflow hood.
5. This step provides uniform apical shoot tips.
6. Shoot tip size and uniformity is an important factor for the success of the protocol.
7. Petri dishes containing the PVS2 solution are placed on ground ice or blocks of sterile ice.
8. Avoid touching the alginate solution with the tip of the micropipette when pouring the calcium chloride solution. Calcium chloride solution will cover the cryo-plate through surface tension.
9. Remove the calcium chloride solution from the cryo-plate, and avoid touching the polymerized gel.
10. Do not use too many cryo-plates or foil strips in a small volume of unloading solution to ensure that the unloading solution temperature does not change. Use 25 mL unloading solution to thaw four cryo-plates or four foil strips.
11. Ammonium is eliminated from recovery media 1 and 2 to reduce explant hyperhydricity.

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