Laboratory Handbook

for

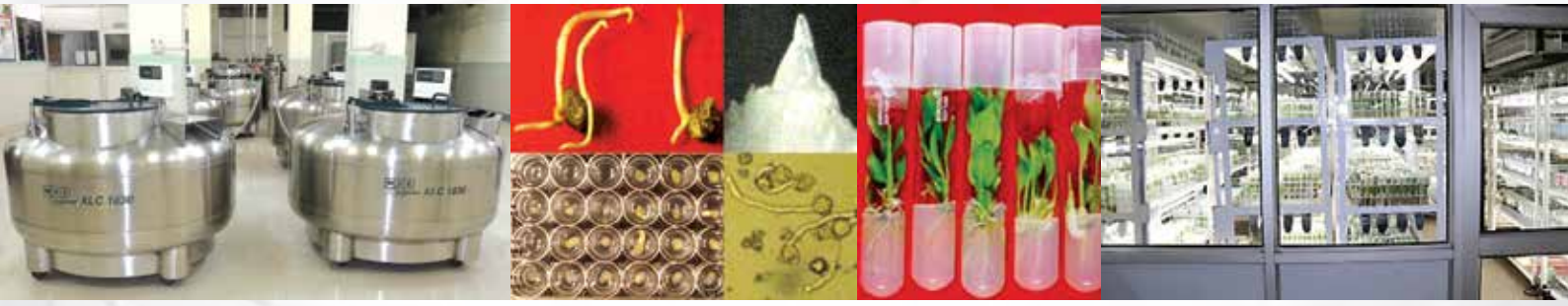
Eighth International Training Course

on

In Vitro and Cryopreservation Approaches for Conservation of Plant Genetic Resources

under the

ICAR-NBPGR – Bioversity International Centre of Excellence on Training



Indian Council of Agricultural Research, National Bureau of Plant Genetic Resources (ICAR-NBPGR)

New Delhi, India

November 5-19, 2019

Laboratory Handbook

for

Eighth International Training Course

on

In Vitro and Cryopreservation Approaches for Conservation of Plant Genetic Resources

under the

ICAR-NBPGR – Bioversity International Centre of Excellence on Training

Indian Council of Agricultural Research, National Bureau of Plant Genetic Resources (ICAR-NBPGR)

New Delhi, India

November 5-19, 2019

Citation

Agrawal A., R. Gowthami, V. Srivastava, E.V. Malhotra, R. Pandey, N. Sharma, S. Gupta, S. Bansal, R. Chaudhury, J.C. Rana, R.K. Tyagi and K. Singh (eds.) (2019) **Laboratory Manual for Eighth International Training Course on *In Vitro* and Cryopreservation Approaches for Conservation of Plant Genetic Resources**. ICAR-National Bureau of Plant Genetic Resources (NBPGR), New Delhi, Bioversity International-India, Delhi, Asia Pacific Association for Agricultural Research Institutions (APAARI)/Asia-Pacific Consortium on Agricultural Biotechnology and Bioresources (APCoAB), Bangkok, Nov. 5-19, 2019, xviii+83 p.

The contents of this manual are collated for the academic purpose of the 'Eighth International Training Course on In Vitro and Cryopreservation Approaches for Conservation of Plant Genetic Resources', held at ICAR-NBPGR, New Delhi, India, from Nov. 5-19, 2019. The information in the manual consists of unpublished as well as published data. Some contents of chapters have been sourced by the authors from their prior publications.

Printed by:

Malhotra Publishing House
B-6, DSIDC Complex, Kirti Nagar, New Delhi - 110015
Ph.: 011-41420246; E-mail: vinay.malhotra@gmail.com

Contents

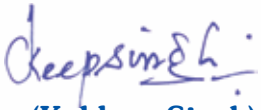
Foreword

The International Training on '*In Vitro* and Cryopreservation Approaches for Conservation of Plant Genetic Resources' organized by ICAR-National Bureau of Plant Genetic Resources (NBPGR) and Bioversity International, India Office, New Delhi, India, during November 5-19, 2019, is the eighth such training under the Center of Excellence (CoE) program approved by Indian Council of Agricultural Research (ICAR). The training is also in collaboration with the Asia-Pacific Association for Agricultural Research Institutes (APAARI) Bangkok, Thailand under its program Asia-Pacific Consortium on Agricultural Biotechnology and Bioresources (APCoAB). This year 22 participants from 13 countries (Algeria, Bangladesh, Chinese Taipei, Fiji, India, Kazakhstan, Madagascar, Samoa, Senegal, Papua New Guinea, Philippines, Ukraine, Uzbekistan) are participating. In the past, more than 100 trainees from nearly 40 countries have benefitted from the training, who subsequently used the skills acquired during these trainings to undertake research work in their own countries, and many of them have become experts in the subject.

The CoE at the Tissue Culture and Cryopreservation Unit (TCCU) of ICAR-NBPGR, New Delhi, was established for organizing regional/international training on a regular basis, to enhance the capacity of national programs for using *in vitro* and cryopreservation techniques for conservation of plant genetic resources (PGR), especially in the developing countries. The CoE is equipped with advanced facilities for *in vitro* conservation and cryopreservation, and expertise with knowledgeable and experienced scientists as resource persons for imparting theoretical knowledge as well as hands-on training. The training provides opportunities for South-South Cooperation as well as North-South collaboration, as faculty are also drawn from other nations like Belgium, Japan, Thailand and UK. The training gives an international visibility to ICAR, for its leadership in capacity development in the area of biotechnology for conservation of PGR.

The present training course is designed for those who are currently involved in the development and use of *in vitro* and/or cryopreservation techniques for the medium- to long-term conservation of germplasm of vegetatively propagated and non-orthodox seed species. The course will consist of a series of lectures with greater emphasis on laboratory practical sessions. It is hoped that this training program will be useful in furthering the cause of conserving precious germplasm of vegetatively propagated and non-orthodox seeded crops, which is an arduous task for the conservation scientists. Also, it would help in fostering networking and partnership amongst the trainers and the trainees, for long-term scientific collaboration at international level.

Organizational support from ICAR Headquarters, Delhi; Bioversity International, India and Central Asia Offices, and APCoAB/APAARI, Bangkok, is sincerely acknowledged. Special thanks to the international faculty who have travelled from far and added value to this course. All colleagues at NBPGR associated with organization and conduct of this training, especially staff of TCCU, are appreciated for their dedication and efforts.



(Kuldeep Singh)
Course Director &
Director, ICAR-NBPGR, New Delhi, India

Preface

This manual is a collection of experimental details of the practicals conducted during the Eighth International Training Course on '*In Vitro* and Cryopreservation Approaches for Conservation of Plant Genetic Resources' organized at the ICAR-National Bureau of Plant Genetic Resources (NBPGR), New Delhi, India during November 5-19, 2019. Information has been provided so that participants can easily carry out the experiments in their own laboratories.

The training course is conducted as per the approved Workplan (2017-21) between Indian Council of Agricultural Research (ICAR), Department of Agricultural Research and Education (DARE) and Bioversity International, Rome/New Delhi, which is under the existing collaborative mechanism as elaborated in Article VII of the Memorandum of Agreement signed in 1996 for scientific and technical cooperation between ICAR and Bioversity International. The ICAR-NBPGR was designated as a Centre of Excellence (CoE) by ICAR and Bioversity International for undertaking international training programmes on '*In Vitro* Conservation and Cryopreservation' of PGR, since 2006, and has undertaken seven such specialized trainings during 2006-2014. The primary aim of the CoE is to provide training opportunities to enhance the capacity of national and international genebanks/laboratories for using advanced biotechnological techniques for conservation of "difficult-to-conserve" germplasm, such as clonally propagated material, recalcitrant seeded species, threatened species, *etc*.

Organization of this training course has been possible due to support of several institutions and individuals. At the outset, sincere gratitude is accorded to Dr Trilochan Mohapatra, Secretary, DARE and DG, ICAR for motivating and facilitating this activity, revived after a gap of nearly five years. Officials at ICAR, especially Dr A.K. Singh, DDG (Crop Science), Dr D.K. Yadava, ADG (Seeds) and Dr A. Arunachalam, ADG (IR), are gratefully acknowledged for their help in various ways. Officials of DARE are thanked for facilitating in obtaining the requisite official permissions, including Mr J. Misra, Mr J.N. Mahto and Ms Juliet Jose. Bioversity International is sincerely thanked for the financial support for logistics of all participants, besides air travel of all external resource persons and few trainees from Central Asia. Staff of Bioversity International office in Delhi are thanked immensely, especially Dr N.K. Krishna Kumar, Regional Coordinator, South and Central Asia; Dr J.C. Rana, GEF National Coordinator; Ms Celine D'Silva, Country Office Manager, India; and Mr Vinay. Most grateful thanks are extended to Dr Ravi Khetarpal, Executive Secretary, APAARI, for agreeing to co-organize this training. Dr Rishi K. Tyagi, Coordinator, APCoAB, has been instrumental in giving wide publicity to the course and facilitating participation of trainees from various countries, thanks to his excellent networking. APCoAB is also thanked for sponsoring the participation of some trainees from Asia-Pacific Region and Mr Vishwanath Sah, Assistant, is acknowledged for his help in arranging their air travel. Importantly, printing of this manual is also very kindly supported by APCoAB.

This year participants from Pacific Region (PNG, Fiji, Samoa), South and East Asia (Bangladesh, India, Philippines, Taiwan), Central Asia (Uzbekistan, Kazakhstan, Ukraine) and Africa (Algeria, Madagascar, Senegal) are beneficiaries from the training course. Grateful thanks are accorded to their respective organizations for nominating and supporting their

candidature. Sincere thanks are extended to Dr Hugh Pritchard (Royal Botanic Gardens, Kew, UK), Dr Bart Panis (Bioversity International, Leuven, Belgium), Dr Takao Niino (Japan) and Dr Kanchit Thammasari (Mahidol University, Bangkok, Thailand) for agreeing to be faculty of the course and spare their valuable time to share their rich experience with the trainees.

Dr Kuldeep Singh, Director, ICAR-NBPGR, has been a pillar of strength and his unflinching support and guidance has been key in organization of this course. He has given freedom in planning and execution of the programme and most grateful thanks are extended to him. All the faculty of the training, committee members, staff of NBPGR (especially TCCU), are gratefully acknowledged, as this training has become a reality only through their efforts and inputs. Since the list is very long and not wanting to risk omission, I am refraining naming individuals! But suffice to state that this training and manual is the outcome of great team effort.

(Anuradha Agrawal)
Course Coordinator &
Officer-In-Charge, TCCU
ICAR-NBPGR, New Delhi, India

Training Schedule

Nov. 5, 2019, Tuesday (Day 1)

Time	Title of Session/Activity	Speaker/ Contact Person(s)
0930 - 1000	Registration of trainees	Rekha Chaudhury and Gowthami R.
1000 - 1130	Inaugural session	
1130 - 1230	***Tea and group photo***	
1230 - 1300	NBPGR Film	Neelam Sharma
1300 - 1400	***Lunch***	
1400 - 1445	**Lecture 1:** *In vitro* conservation and cryopreservation activities at ICAR-NBPGR	Anuradha Agrawal
1445 - 1600	Visit to various laboratories of ICAR-NBPGR	Sandhya Gupta and Vartika Srivastava
1600 - 1615	***Tea***	
1615 - 1700	**Lecture 2:** Role of APCoAB in promoting application of biotechnology and capacity development in Asia-Pacific	R.K. Tyagi

Nov. 6, 2019, Wednesday (Day 2)

0930 - 1015	**Lecture 3:** Orchid micropropagation and cryopreservation for conservation of plant genetic resources - The Thai experience	Kanchit Thammasari
1015 - 1100	**Practical Session 1:** Group A Preparation of cryopreservation solutions and media	Era V. Malhotra, Gowthami R., D.K. Nerwal, Ramesh Chandra
	Practical Session 2: Group B Isolation of shoot tips/meristems in monocot and dicots	Anuradha Agrawal, Neelam Sharma, D.P.S. Meena
1100 - 1115	***Tea***	
1115 - 1300	**Practical Session 1:** Group A **Practical Session 2:** Group B	
1300 - 1400	***Lunch***	

Time	Title of Session/Activity	Speaker/ Contact Person(s)
1400 - 1600	**Practical Session 1:** Group B **Practical Session 2:** Group A	
1600 - 1615	***Tea***	
1615 - 1700	**Lecture 4:** Information Management Systems for PGR	Sunil Archak

Nov. 7, 2019, Thursday (Day 3)

0930 – 1015	**Lecture 5:** *In vitro* cryopreservation techniques – The know-how	Neelam Sharma
1015 - 1100	**Lecture 6:** Principles of cryopreservation and practical aspects of cryobanking	Rekha Chaudhury
1100 - 1115	***Tea***	
1115 - 1300	**Practical Session 3:** Group A Handling of fruits/seeds of non-orthodox species prior to cryopreservation	Rekha Chaudhury, Sangita Bansal, A.P. Singh
	Practical Session 4: Group B Cryopreservation using vitrification technique	Neelam Sharma, Gowthami R., Ramesh Chandra
1300-1400	***Lunch***	
1400 - 1530	**Practical Session 3:** Group B **Practical Session 4:** Group A	
1530 - 1545	***Tea***	
1545 - 1615	**Practical Session 3:** Group B **Practical Session 4:** Group A	
1615 - 1700	**Lecture 7 :** CWR conservation for abiotic and biotic stress mitigation in horticulture crops	N.K. Krishna Kumar

Nov. 8, 2019, Friday (Day 4)

0930 – 1015	**Lecture 8:** Indian PGR management system	Kuldeep Singh
1015 – 1045	**Lecture 9:** New vitrification-based cryotechniques (V-cryoplate and D-cryoplate)	Takao Niino
1045 – 1100	***Tea***	
1100 – 1300	**Practical Session 5:** Group A V-cryoplate and D-cryoplate techniques	Takao Niino, Sandhya Gupta, D.P.S. Meena
	Practical Session 6: Group B Cryopreservation of budwoods	Vartika Srivastava, Sangita Bansal, D.K. Nerwal

Time	Title of Session/Activity	Speaker/ Contact Person(s)
1300 – 1400	***Lunch***	
1400 – 1600	**Practical Session 5:** Group A **Practical Session 6:** Group B	
1600- 1615	***Tea***	
1615 – 1700	**Practical Session 5:** Group A **Practical Session 6:** Group B	

Nov. 9, 2019, Saturday (Day 5)

0930 – 1015	**Lecture 10:** Dormant buds cryopreservation – Theory and practice	Vartika Srivastava
1015 - 1100	**Lecture 11:** *In vitro* conservation and cryopreservation in temperate fruit crops	Sandhya Gupta
1100 – 1115	***Tea***	
1115 - 1300	**Practical Session 5:** Group B V-cryoplate and D-cryoplate techniques	Takao Niino, Sandhya Gupta, D.P.S. Meena
	Practical Session 6: Group A Cryopreservation of budwoods	Vartika Srivastava, Sangita Bansal, D.K. Nerwal
1300 - 1400	***Lunch***	
1400 - 1700	**Practical Session 5:** Group B **Practical Session 6:** Group A	
1530 - 1545	***Tea***	

Nov. 10, 2019, Sunday (Day 6)

0930 - 1500	Holiday - City Trip	

Nov. 11, 2019, Monday (Day 7)

0930 - 1045	**Lecture 12:** Cryobiotechnology of seeds – Fundamental aspects	Hugh Pritchard
1045 - 1100	***Tea***	
1100 - 1300	**Practical Session 7:** Group A Cryopreservation of non-orthodox seeds using varied techniques	Hugh Pritchard, Rekha Chaudhury, A.P. Singh
	Practical Session 8: Group B Cryopreservation using encapsulation-dehydration technique	Sandhya Gupta, Era V. Malhotra, D.K. Nerwal, Suresh Mali

Time	Title of Session/Activity	Speaker/ Contact Person(s)
1300 - 1400	***Lunch***	
1400 - 1600	**Practical Session 7:** Group A **Practical Session 8:** Group B	
1600- 1615	***Tea***	
1615 - 1700	Crop genetic resources - International conservation and utilization status	J.C. Rana

Nov. 12, 2019, Tuesday (Day 8)

0930 - 1045	**Lecture 13:** Cryobiotechnology of seeds – Achievements and prospects	Hugh Pritchard
1045 - 1100	***Tea***	
1100 - 1300	**Practical Session 7:** Group B Cryopreservation of non-orthodox seeds using varied techniques	Hugh Pritchard, Rekha Chaudhury, A.P. Singh
	Practical Session 8: Group A Cryopreservation using encapsulation-dehydration technique	Sandhya Gupta, Era V. Malhotra, D.K. Nerwal, Suresh Mali
1300 - 1400	***Lunch***	
1400 - 1600	**Practical Session 7:** Group B **Practical Session 8:** Group A	
1600 - 1615	***Tea***	
1615 - 1700	**Lecture 14:** Cryobanking germplasm from stressed ecosystem - Indian experience	S.K. Malik

Nov. 13, 2019, Wednesday (Day 9)

0930 - 1015	**Lecture 15:** Pollen cryopreservation - an aid for plant breeding	Gowthami R.
1015 - 1100	**Practical Session 9:** Group A *Musa* cryopreservation using varied explant systems	Anuradha Agrawal, D.P.S. Meena
	Practical Session 10: Group B Cryopreservation of pollen	Gowthami R., Vartika Srivastava, A.P. Singh
1100 - 1115	***Tea***	
1115 - 1300	**Practical Session 9:** Group A **Practical Session 10:** Group B	
1300 - 1400	***Lunch***	
1400 - 1630	**Practical Session 9:** Group A **Practical Session 10:** Group B	

Time	Title of Session/Activity	Speaker/ Contact Person(s)
1630 - 1645	***Tea***	
1645 - 1700	Presentation by trainees	Sangita Bansal

Nov. 14, 2019, Thursday (Day 10)

0930 - 1015	**Lecture 16:** *In vitro* conservation and cryopreservation in tuber crops	Sangita Bansal
1015 - 1030	***Tea***	
1030 - 1300	**Practical Session 9:** Group B *Musa* cryopreservation using varied explant systems	Anuradha Agrawal, D.P.S. Meena
	Practical Session 10: Group A Cryopreservation of pollen	Gowthami R., Vartika Srivastava, A.P. Singh
1300 - 1400	***Lunch***	
1400 - 1600	**Practical Session 9:** Group B **Practical Session 10:** Group A	
1600 - 1615	***Tea***	
1615 - 1700	Presentation by trainees	Sandhya Gupta

Nov. 15, 2019, Friday (Day 11)

0930 - 1015	**Lecture 17:** Genetic stability studies in *in vitro* conserved and cryoconserved germplasm	Era V. Malhotra
1015 - 1030	***Tea***	
1030 - 1300	**Practical Session 11:** Group A Cryopreservation in alliums	Gowthami R., Ramesh Chandra
	Practical Session 12: Group B DNA isolation, purification and quantification	Sangita Bansal, Era V. Malhotra, Suresh Mali
1300 - 1400	***Lunch***	
1400 - 1600	**Practical Session 11:** Group B **Practical Session 12:** Group A	
1600 - 1615	***Tea***	
1615 - 1700	Presentation by trainees	Gowthami R.

Nov. 16, 2019, Saturday (Day 12)

0930 - 1015	**Lecture 18:** Health testing of germplasm conserved in *in vitro* genebanks and cryogenebanks	S.C. Dubey

Time	Title of Session/Activity	Speaker/ Contact Person(s)
1015 - 1030	***Tea***	
1030 - 1300	**Practical Session 13:** Group A Virus indexing of *in vitro* germplasm	Celia Chalam V., Ashok Maurya
	Practical Session 14: Group B PCR and gel analyses of SSR and ISSR markers for genetic stability studies	Era V. Malhotra, Sangita Bansal, Suresh Mali
1300 - 1400	***Lunch***	
1400 - 1600	**Practical Session 13:** Group A **Practical Session 14:** Group B	
1600 - 1615	***Tea***	
1615 - 1700	Interactive Session	

Nov. 17, 2019, Sunday (Day 13)

0930 - 1500	Holiday - City Trip	

Nov. 18, 2019, Monday (Day 14)

0930 - 1100	**Lecture 19:** How to cryoconserve new plant species?	Bart Panis
1100 - 1115	***Tea***	
1115 - 1300	**Practical Session 13:** Group B Virus indexing of *in vitro* germplasm	Celia Chalam V., Ashok Maurya
	Practical Session 14: Group A PCR and gel analyses of SSR and ISSR markers for genetic stability studies	Era V. Malhotra, Sangita Bansal, Suresh Mali
1300 - 1400	***Lunch***	
1400 - 1600	**Practical Session 13:** Group B **Practical Session 14:** Group A	
1600 - 1615	***Tea***	
1615 - 1700	Feedback by trainees	Neelam Sharma

Nov. 19, 2019, Tuesday (Day 15)

0930 - 1100	**Lecture 20:** *In Vitro* Genebanks and Cryogenebanks –Global Status and Prospects	Bart Panis
1100 - 1115	***Tea***	
1115-1300	Valedictory Function	

List of Experts & Trainers

Course Director

Dr Kuldeep Singh
Director
ICAR-National Bureau of Plant Genetic Resources, Pusa Campus, New Delhi - 110012, INDIA
director.nbpgr@icar.gov.in

International Faculty

Dr Ir. Bart Panis
Senior Researcher
Bioversity International, Leuven, Belgium
bart.panis@biw.kuleuven.be

Prof. Hugh Pritchard
Head of Research
Senior Research Leader, Royal Botanic Gardens,
Kew, United Kingdom
h.pritchard@kew.org

Dr Kanchit Thammasiri
Associate Professor
Dept of Plant Science, Mahidol University,
Bangkok, Thailand
kanchit.tha@mahidol.ac.th

Dr Takao Niino
653 Showa, Shinjo, Yamagata, Japan
niino.takao.fp@alumni.tsukuba.ac.jp

Dr N.K. Krishna Kumar
Regional Representative,
South & Central Asia,
Bioversity International-India
k.kumar@cgiar.org

Dr J.C. Rana
National Coordinator,
UN GEF Project
Bioversity International-India
j.rana@cgiar.org

Dr R.K. Tyagi
Coordinator APCoAB, APAARI
Bangkok, Thailand
rishi.tyagi@apaari.org

Faculty, Tissue Culture and Cryopreservation Unit, ICAR-NBPGR

Dr Anuradha Agrawal
Officer-In-Charge
anuradha.agrawal@icar.gov.in

Dr Rekha Chaudhury
Principal Scientist
rekha.chaudhury@icar.gov.in

Dr Ruchira Pandey
Principal Scientist
ruchira.pandey@icar.gov.in

Dr Neelam Sharma
Principal Scientist
neelam.sharma1@icar.gov.in

Dr Sandhya Gupta
Principal Scientist
sandhya.gupta@icar.gov.in

Dr Sangita Bansal
Principal Scientist
sangita.bansal@icar.gov.in

Dr Vartika Srivastava
Scientist
vartika.srivastava@icar.gov.in

Dr Era Vaidya Malhotra
Scientist
era.vaidya@icar.gov.in

Dr Gowthami R.
Scientist
gowthami.r@icar.gov.in

Faculty, ICAR and NBPGR

Dr Sunil Chandra Dubey
Head of Division, Division of Plant Quarantine (DPQ)
sunil.dubey@icar.gov.in

Dr Celia Chalam Vasimalla
Principal Scientist, DPQ
celia.chalam(@icar.gov.in

Dr Sunil Archak
Officer-In-Charge, Agriculture Knowledge Management Unit
sunil.archak@icar.gov.in

Dr. S.K. Malik
Scientific Officer to DG, ICAR
surendra.malik@icar.gov.in

Technical Staff, ICAR-NBPGR

Mr. Ashok Maurya
Chief Technical Officer
ashok.maurya@icar.gov.in

Mr. D.K. Nerwal
Assist. Chief Tech. Officer
devender.nerwal@icar.gov.in

Mr. A.P. Singh
Assist. Chief Tech. Officer
anang.pal@icar.gov.in

Mr. D.S. Meena
Senior Tech. Assist.
dharmpal.meena@icar.gov.in

Mr. Ramesh Chandra
Technical Officer
ramesh.chandra4@icar.gov.in

Mr. Satya Prakash
Technical Officer
satya.prakash1@icar.gov.in

Mr. Suresh Chand Mali
Technical Assistant
suresh.mali@icar.gov.in

Research Fellows, ICAR-NBPGR

Ms. Shivani Singh
Senior Research Fellow
shivani.s.2693@gmail.com

Ms. Rishu Jain
Junior Research Fellow
rishujain512512@gmail.com

Mr. Hardeep Singh
Junior Research Fellow
hardeepsinghh16@gmail.com

Mr. Vikrant
Junior Research Fellow
vikrantgautam91@gmail.com

Ms. Kanika Sharma
Junior Research Fellow
kanika2794@gmail.com

List of Trainees

Abdullah Mohammad Shohael — **Bangladesh**
Professor
Department of Biotechnology & Genetic Engineering
Jahangirnagar University, Dhaka, Savar-1342
Bangladesh
sohaelam@yahoo.com

Aliou Ndiaye — **Senegal**
Assistant Professor
University of Cheikh Anta DIOP
Senegal, Dakar Fann-5005
Dakar, Senegal
aliou.ndiaye@ucad.edu.sn

Bakoarimalala Faratiana Randriamahaleo — **Madagascar**
Student
University of Antananarivo
Ambohitsaina-101, Madagascar
faratianarandriamahaleo@gmail.com

Boualem Harfi — **Algeria**
Research Scientist
Biotechnology Research Center (C.R.Bt)
Ali Mendjeli, Constantine-25000
Algeria
b.harfi@crbt.dz

Chung-Yi Lin — **Chinese Taipei**
Project Specialist
Taiwan Banana Research Institute
No. 1, Rongquan St., Jiuru Township
Pingtung-904, Taiwan (R.O.C.)
Icy@mail.banana.org.tw

Ghanshyam Bhaurao Patil — **India**
I/C Associate Research Scientist & Head
Centre for Advanced Research in Plant
Tissue Culture, Anand Agricultural University,
Gujarat-388110
gbpatil@aau.in

H. Kencharaddi — **India**
Research Associate
Bioversity International,
Post Office, Bengaluru-560065
hgkencharaddi@gmail.com

Irelynn Valovalo Laban — **Samoa**
Tissue Culture Officer (PGR)
Ministry of Agriculture & Fisheries,
Crops Division, Nu'u Apia, Samoa
irelynn.laban@gmail.com

Ju-Ling Lin — **Chinese Taipei**
Assistant Researcher
Taiwan Seed Improvement and Propagation Station,
Council of Agriculture, Executive Yuan, No. 6, Xingzhong St., Xinshe Dist., Taichung-42644, Taiwan, (R.O.C.)
jllin@tss.gov.tw

Kuan-Te Li — **Chinese Taipei**
Associate Research Fellow
Agricultural Technology Research Institute
Plant Technology Laboratories,
No. 1, Ln. 51, Dahu Rd. Hsinchu-300, Taiwan
kli@mail.atri.org.tw

Lerma San Jose Maldia — **Philippines**
Assistant Professor
University of the Philippines Los Banos,
College of Forestry and Natural Resources, Department of Forest Biological Sciences, Laguna, Los Banos-4031
Philippines
ismaldia@up.edu.ph

Maksat Meirambekuly Nurmanov — **Kazakhstan**
Junior Researcher
Institute of Plant Biology and Biotechnology
050040, Kazakhstan,
Almaty, Timiryazev Street, 45
nurmanovmaksat@mail.ru

Manjunath Girigowda — **India**
Assistant Professor
University of Horticultural Sciences,
Bagalkot Regional Horticultural Research and Extension Centre, Bengaluru, College of Horticulture campus
GKVK Post, Bengaluru-560065, Karnataka
gmanjunath2007@gmail.com

Maraia Dure Ravulo — **Fiji**
Laboratory Technician
The Pacific Community,
Centre for Pacific Crops and Trees,
Vatoa Road, Narere, Fiji Central Suva, Fiji
maraiar@spc.int

Nazgul Kabdullakyzy Rymkhanova — **Kazakhstan**
Junior Researcher
Institute of Plant Biology and Biotechnology
050040, Kazakhstan,
Almaty, Timiryazev Street, 45
nazka_0993@mail.ru

Rostyslav Yaroslavovych Blium — **Ukraine**
Student/Laboratory Assistant
Taras Shevchenko National University of Kyiv,
Educational and Scientific Center
"Institute of Biology and Medicine",
Hlushkova Avenue, 2, Kyiv, 03127, Ukraine
blume.rostislav@gmail.com

Salomat Khudaybergenovna Abduramnova — **Uzbekistan**
Head, *In vitro* Lab
Scientific Research Institute of Horticulture, Viticulture and Winemaking
Gulistan Village, Tashkent Region, Tashkent District-111116, Uzebkistan
abduramanova28@mail.ru

Sandjar Saydazimovich Buzurukov — **Uzbekistan**
Researcher
Institute of Genetics and Plant Experimental Biology,
Academy Of Sciences of Uzbekistan, Tashkent Region,
Kibray-111226, Uzbekistan
sai-baboev@yandex.ru

Sarrah Mamonjiharisoa Randremanana — **Madagascar**
Ph.D. Student
Department of Plant Biology and Ecology,
University of Antananarivo
PO Box 906, Madagascar
harisoasah@yahoo.fr

Thiruvengadam Venkatesan — **India**
Assistant Professor
Department of Plant Genetic Resources
Centre for Plant Breeding and Genetics
Tamil Nadu Agricultural University
Coimbatore-641003, Tamil Nadu
thirugen@gmail.com

Ulamila Duguci Lutu **Fiji**
Research Technician
The Pacific Community (SPC),
Centre for Pacific Crops and Trees,
FNTC 2 Road, Narere, Fiji,
Central Suva, Fiji
ulamilal@spc.int

Wilfred Lofuko Wau **Papua New Guinea**
Tissue Culture Laboratory Manager/Plant Pathologist
National Agriculture Research Institute (NARI)
Highlands Regional Centre PO Box 210,
Ukarumpa Eastern Highlands Province
Papua New Guinea (PNG)
Wilfred.wau@nari.org.pg

The Organizers

Indian Council of Agricultural Research (ICAR)

http://www.icar.gov.in

The ICAR is an autonomous organisation under the Department of Agricultural Research and Education (DARE), Ministry of Agriculture and Farmers Welfare, Government of India. Formerly known as Imperial Council of Agricultural Research, it was established on 16 July 1929 as a registered society under the Societies Registration Act, 1860 in pursuance of the report of the Royal Commission on Agriculture. The ICAR has its headquarters at New Delhi. The Council is the apex body for co-ordinating, guiding and managing research and education in agriculture including horticulture, fisheries and animal sciences in the entire country. With 101 ICAR institutes and 71 agricultural universities spread across the country this is one of the largest national agricultural systems in the world. The ICAR has played a pioneering role in ushering Green Revolution and subsequent developments in agriculture in India through its research and technology development that has enabled the country to increase the production of foodgrains by 5.4 times, horticultural crops by 10.1 times, fish by 15.2 times, milk 9.7 times and eggs 48.1 times since 1951 to 2017, thus making a visible impact on the national food and nutritional security. It has played a major role in promoting excellence in higher education in agriculture. It is engaged in cutting edge areas of science and technology development and its scientists are internationally acknowledged in their fields.

Bioversity International

https://www.bioversityinternational.org

Bioversity International is a global research-for-development organization, and one of the 15 Research Centres of the Consultative Group for International Agricultural Research (CGIAR), a global research partnership for a food-secure future. The vision of Bioversity International is that agricultural biodiversity nourishes people and sustains the planet. It delivers scientific evidence, management practices and policy options to use and safeguard agricultural and tree biodiversity to attain sustainable global food and nutrition security. Bioversity International works with partners in low-income countries in different regions where agricultural and tree biodiversity can contribute to improved nutrition, resilience, productivity and climate change adaptation.

Asia-Pacific Association of Agricultural Research Institutions (APAARI)

http://www.apaari.org

The APAARI, with its headquarters in Bangkok, is a unique voluntary, membership-based, self-mandated, apolitical and multi-stakeholder regional organization in the Asia-Pacific region. It promotes and strengthens agriculture and agri-food research and innovation systems through

partnerships and collaboration, capacity development and advocacy for sustainable agricultural development in the region. Since its establishment in 1990, APAARI has significantly contributed towards addressing agricultural research needs and enhancing food and nutritional security in the region. The close links, networks, partnerships and collaboration with stakeholders that APAARI has developed over the years, as well as its goodwill, authority and focus on results, make the Association an important actor in the region. The ultimate aim of APAARI is to help realising sustainable development goals in Asia and the Pacific.

Asia-Pacific Consortium on Agricultural Biotechnology and Bioresources (APCoAB)

http://www.apaari.org/web/our-projects/apcoab/

The APCoAB, established in 2003 under the umbrella of APAARI, has the mission to harness the benefits of agricultural biotechnology and bioresources for human and animal welfare through the application of latest scientific technologies while safeguarding the environment for the advancement of society in the Asia-Pacific region. APCoAB's main objectives are to (i) serve as neutral forum for the key partners engaged in research, development, commercialization and education/learning of agricultural biotechnology as well as environmental safety in the Asia-Pacific region; (ii) Application of biotechnological tools for bioprospecting, conservation and sustainable use of bioresources; (iii) facilitate and promote the process of greater public awareness and understanding relating to important issues of IPRs, *sui generis* systems, biosafety, risk assessment, harmonization of regulatory procedures, and access and benefit sharing in order to address various concerns relating to adoption of agricultural biotechnology and sustainable use of bioresources; and (iv) facilitate human resource development for meaningful application of agricultural biotechnology and use of bioresources to enhance sustainable agricultural productivity, as well as product quality, for the welfare of both farmers and consumers.

ICAR-National Bureau of Plant Genetic Resources (NBPGR)

The NBPGR is a nodal institute under ICAR to undertake research, education and service activities in managing plant genetic and genomic resources, for sustainable growth of agriculture. Established in the year 1976, NBPGR has its headquarters at New Delhi, India and has 10 Regional Stations across the country. Its mandate include management and promotion of sustainable use of plant genetic and genomic resources of agri-horticultural crops and to carry out related research, coordination and capacity building in PGR management and policy issues governing access and benefit sharing of their use and molecular profiling of varieties of agri-horticultural crop and GM detection technology research. NBPGR has the National Genebank Bank, the second largest plant germplasm bank in the world, comprising Seed Genebank, *In Vitro* Genebank and Cryogenebank with >0.45 million accessions. The expertise at Tissue Culture and Cryopreservation Unit (TCCU) in the *in vitro* conservation and cryopreservation technology has led to recognition of NBPGR as a Centre of Excellence (CoE) in Training in these areas by ICAR and Bioversity International.

Organizing Committees

International Advisory Committee

1. **Dr T. Mohapatra**, ICAR, New Delhi
2. **Dr N.K. Krishna Kumar**, Bioversity International, New Delhi
3. **Dr Ravi Khetarpal**, APAARI, Bangkok
4. **Dr R.K. Tyagi**, APCoAB, Bangkok
5. **Dr J.C. Rana**, Bioversity International, New Delhi

Course Director

1. **Dr Kuldeep Singh**, Director, ICAR-NBPGR

Course Coordinator

1. **Dr Anuradha Agrawal**, Principal Scientist & Officer-In-Charge, TCCU

Course Co-Coordinator

1. **Dr S.K. Kaushik**, Principal Scientist, DGE & Nodal Officer, HRD

Course Convenors

1. **Dr Neelam Sharma**, Principal Scientist, TCCU
2. **Dr Sandhya Gupta**, Principal Scientist, TCCU
3. **Dr Sangita Bansal**, Principal Scientist, TCCU
4. **Dr Rekha Chaudhury**, Principal Scientist, TCCU
5. **Dr Ruchira Pandey**, Principal Scientist, TCCU

Course Co-Convenors

1. **Dr Vartika Srivastava**, Scientist, TCCU
2. **Dr Era Vaidya Malhotra**, Scientist, TCCU
3. **Dr Gowthami R.**, Scientist, TCCU

Registration Committee

1.	**Dr Rekha Chaudhury**, Principal Scientist, TCCU	Chair
2.	**Dr Gowthami R.**, Scientist, TCCU	Co-Chair
3.	**Mr Anang Pal Singh**, ACTO, TCCU	Member

4.	**Ms Kanchan Khurana**, Private Secretary, Director Cell	Member
5.	**Ms Geeta Devi**, SSS, TCCU	Member

Accommodation and Transport Committee

1.	**Dr Neelam Sharma**, Principal Scientist, TCCU	Chair
2.	**Dr Sandhya Gupta**, Principal Scientist, TCCU	Co-Chair
3.	**Dr Amit Kumar**, Senior Scientist, DGR	Member
4.	**Mr Surender Singh Ranga**, CTO, GEPU	Member
5.	**Mr D.K. Nerwal**, ACTO, TCCU	Member
6.	**Mr Ramesh Chandra**, Technical Officer, TCCU	Member
7.	**Mr Nand Kishore**, SSS, TCCU	Member

Food and Refreshment Committee

1.	**Dr Sandeep Kumar**, Principal Scientist, DGE	Chair
2.	**Dr Vartika Srivastava**, Scientist, TCCU	Co-Chair
3.	**Dr Pradeep Kumar**, Scientist, DPQ	Member
4.	**Mr D.P.S. Meena**, Senior Technical Officer, TCCU	Member
5.	**Mr Suresh Mali**, Technical Assistant, TCCU	Member
6.	**Ms Geeta Devi**, SSS, TCCU	Member

Hall Arrangement Committee

1.	**Dr Sandhya Gupta**, Principal Scientist, TCCU	Chair
2.	**Dr Era Vaidya Malhotra**, Scientist, TCCU	Co-Chair
3.	**Mr N.S. Panwar**, CTO, DPEGC	Member
4.	**Mr Arun Kumar Sharma**, Technical Officer, Director Cell	Member
5.	**Mr Vijay Kumar Mandal**, Technical Assistant, AKMU	Member
6.	**Mr Abhay Sharma**, Photographer, DGC	Member
7.	**Mr Hari Chand Paswan**, SSS, Director Cell	Member
8.	**Mr Surinder Kumar**, SSS, Director Cell	Member

Laboratory Arrangement Committee

1.	**Dr Sangita Bansal**, Principal Scientist, TCCU	Chair
2.	**Dr Gowthami R.**, Scientist, TCCU	Co-Chair
3.	**Mr D.K. Nerwal**, ACTO, TCCU	Member
4.	**Mr Anang Pal Singh**, ACTO, TCCU	Member
5.	**Mr D.P.S. Meena**, Senior Technical Officer, TCCU	Member
6.	**Mr Satya Prakash**, Technical Officer, DGC	Member
7.	**Mr Ramesh Chandra**, Technical Officer, TCCU	Member

8.	**Mr Suresh Mali**, Technical Assistant, TCCU	Member
9.	**Mr Chandeshwar Rai**, SSS, TCCU	Member

Publication Committee

1.	**Dr Ruchira Pandey**, Principal Scientist, TCCU	Chair
2.	**Dr Anuradha Agrawal**, Principal Scientist, TCCU	Co-Chair
3.	**Dr Vartika Srivastava**, Scientist, TCCU	Member
4.	**Dr Era Vaidya Malhotra**, Scientist, TCCU	Member
5.	**Dr Gowthami R.**, Scientist, TCCU	Member

Purchase and Finance Committee

1.	**Dr K.K. Gangopadhyay**, Principal Scientist, DGE	Chair
2.	**Dr Sangita Bansal**, Principal Scientist, TCCU	Co-Chair
3.	**Dr Era Vaidya Malhotra**, Scientist, TCCU	Member
4.	**Mr Satya Prakash**, Technical Officer, DGC	Member
5.	Sr. Administrative Officer/ Nominee	Member
6.	Sr. Finance & Accounts Officer/ Nominee	Member

1. Preparation of solutions and media for cryopreservation

Era Vaidya Malhotra, Gowthami R., D.K. Nerwal and Ramesh Chandra

Introduction

Cryopreservation is utilized as an efficient method for long-term conservation of clonally propagated crops, as at -196°C, *i.e.,* the temperature of liquid nitrogen (LN), all cellular, metabolic and biochemical reactions of cells some to a standstill, and the plant material can be stored without any changes or deterioration for extended time periods. Cryopreservation is based on avoidance of intracellular ice crystal formation during rapid cooling in liquid nitrogen, as they cause irreversible damage to cell membranes thereby destroying their semi permeability. The concept revolves around the dehydrating the cells followed by vitrification of intercellular water directly to an amorphous state, avoiding any intracellular freezing. To achieve this, a concentrated cellular solution is desired followed by rapid freezing, which is achieved by air drying, freeze dehydration, application of penetrating or non-penetrating substances or acclimation.

Generally, vitrification is achieved by direct immersion in LN after exposing the cells to extremely concentrated (7 to 8 M) cryoprotectant solutions. Cryoprotectants are chemicals utilized to protect tissues under conditions of exposure to ultra-low temperatures. A variety of chemicals, such as, sucrose, glucose, proline, mannitol, glycerol, sorbitol, trehalose, polyethylene glycol and ethylene glycol, may be used as cryoprotectants. These may be applied in combination with DMSO and/or three component mixtures, within the concentration range of 5–15% (w/v or v/v) or 0.5–1.0 M. Vitrification solutions contain two or more of the above-mentioned components and applied either at low (~0°C) or at ambient (~25°C) conditions and at varying concentrations.

To develop a reproducible cryopreservation protocol several solutions need to be prepared with accuracy. The various solutions required in different procedures are described below.

Preparation of preculture medium

Preculture medium is used to acclimatize the explants to higher osmoticum before exposing them to cryoprotectant solutions. The preculture medium contains a dehydrating agent, usually mannitol, sucrose or sorbitol, in the range of 0.3–1 M in MS (Murashige and Skoog, 1962) basal medium (semi-solid or liquid).

For preparing 1l of preculture medium:

1. Add the required volume of MS stock solutions (Table 1), in a flask.

2. Dissolve the desired amount of sucrose completely.
3. Make up the volume 1000 ml using double distilled water and adjust the pH to 5.8. Add 7-8 g of agar-agar (for semi-solid medium) and dissolve the agar at high temperature (using gas stove or microwave oven).
4. Dispense the medium in glass bottles and autoclave at 15 psi at 121°C for 15 min.
5. Allow the medium to cool to room temperature, dispense into plastic Petri plates (60 or 90 mm diameter, as per requirement) and allow to solidify.
6. Seal the Petri plates and store them for further use.

Preparation of vitrification solutions

Plant Vitrification Solution 2 (PVS2)

The plant vitrification solution PVS2 developed by Sakai *et al*. (1990) comprises 30% (w/v) glycerol, 15% (w/v) ethylene glycol, 15% (w/v) DMSO and 0.4 M sucrose in MS medium. The pH is adjusted to 5.8.

For 100 ml of the solution, the volume of the respective components is:

Glycerol (MW=92.09) 30%	-	30 ml
Ethylene Glycol (MW=67.02) 15%	-	15 ml
DMSO (MW=78.13) 15%	-	15 ml

1. Add the required volume of MS stock solutions (Table 1), in a 250 ml flask.
2. Dissolve sucrose at the concentration of 0.4 M (136.92 g/l) and stir to dissolve it completely.
3. Add 15 ml DMSO and stir well.
4. Add 15 ml Ethylene Glycol and stir well.
5. Slowly add 30 ml Glycerol while stirring to dissolve completely.
6. Make up the volume to 100 ml with distilled water and adjust the pH to 5.8.
7. Autoclave or filter-sterilize the solution and store at low temperature (in a freezer < 0°C) till further use.

Plant Vitrification Solution 3 (PVS3)

Developed by Nishizawa *et al*. (1993), PVS3 solution contains 50% (w/v) sucrose and 50% (w/v) glycerol in liquid MS medium. For 100 ml of the solution:

1. Add the required volume of MS stock solutions (Table 1) in a 250 ml flask.
2. Dissolve 50 g sucrose and stir to dissolve it completely.
3. Add 50 ml glycerol while stirring to dissolve completely.
4. Make up the volume to 100 ml with distilled water and adjust the pH to 5.8/
5. Autoclave or filter-sterilize the solution and store at low temperature (in a freezer < 0°C) till further use.

Loading solution (LS)

It usually comprises 2 M glycerol in 0.4 M sucrose MS medium. To prepare 100 ml of loading solution:

1. Dissolve 13.68 g/100 ml sucrose (0.4 M) in liquid MS medium.
2. Add 18.42 ml glycerol while stirring to dissolve completely.
3. Make up the volume to 100 ml, adjust the pH to 5.8.
4. Autoclave or filter-sterilize the solution and store at low temperature (in a freezer < 0°C) till further use.

Recovery Solution (RS) or Unloading Solution (US)

It usually comprises 1.2 M sucrose MS medium. To prepare 100 ml of RS/US:

1. Dissolve 41.08 g/100 ml sucrose (1.2 M) in liquid MS medium.
2. Make up the volume to 100 ml, adjust the pH to 5.8.
3. Autoclave or filter-sterilize the solution and store at low temperature (in a freezer < 0°C) till further use.

Preparation of solutions for encapsulation – dehydration technique

Sodium alginate solution (3%)

For preparing 100 ml of alginate solution with 0.5 M sucrose:

1. Add the required volume of MS stock solutions, except halides (Table 1), in a flask.
2. Dissolve 17.1 g/100 ml of sucrose (0.5 M) completely.
3. Make up the volume 50 ml using double distilled water and adjust the pH to 5.8.
4. Place the solution on a magnetic stirrer cum hot plate.
5. To dissolve 3 g of sodium alginate in the above solution; add in small quantities with constant stirring, till it dissolves completely.
6. Transfer the solution to a 250 ml bottle, autoclave and store for further use.

Calcium chloride solution (100 mM)

For 500 ml of $CaCl_2$ solution (100 mM):

1. Dissolve 7.35 g of calcium chloride in liquid MS medium, without halides (Table 1).
2. Make up the volume to 500 ml using double distilled water and adjust pH to 5.8.
3. Dispense the solution in two bottles of 250 ml each, autoclave and store for use.

Osmotic dehydration solution (0.75 M sucrose)

For 100 ml of this solution:

1. Add the desired amount of all the MS stock solutions (Table 1) in a flask.
2. Dissolve 25.6 g sucrose in the above solution.
3. Make up the volume to 100 ml using double distilled water and adjust the pH to 5.8.
4. Autoclave the solution and store for future use.

Note : Cryoprotectants and pre-culture additives must be of high purity, use of analytical grade sucrose and spectroscopically pure DMSO is recommended.

Table 1. Constituents for stock solution preparation for Murashige & Skoog (MS) Basal medium

	Chemicals	Conc. of stock solution (×100)	Amount of stock per litre of medium
A.	Phosphate borate molybdate KH_2PO_4 H_3BO_3 $Na_2MoO_4.2H_2O$	(1000 ml) 17.0 g 0.62 g 25 mg	10 ml
B.	Sulphate $MgSO_4.7H_2O$ $MnSo_4.4H_2O$ $ZnSo_4.7H_2O$ $CuSo_4.5H_2O$	(1000 ml) 37.0 g 1.69 g 0.86 g 2.5 mg	10 ml
C.	**Halides** $CaCl_2.2H_2O$ KI $CoCl_2.6H_2O$	(1000 ml) 44.0 g 83.0 mg 2.5 mg	10 ml
D.	**Nitrate** NH_4NO_3 KNO_3	(1000 ml) 165.0 g 190.0 g	10 ml
E.	**Iron** Na_2 EDTA.$2H_2O$ $FeSo_4.7H_2O$	(1000 ml) 3.73 g 2.78 g	10 ml
F.	**Vitamins** Pyridoxine.HCl Nicotinic acid Thiamine.HCl Glycine	(100 ml) 50.0 mg 50.0 mg 20.0 mg 0.2 g	1 ml

***Note*:** All the constituents are dissolved in 250 ml distilled water separately and volume made up to 1000 m;. 10ml of stock is used per litre of the medium.

Plant growth regulators like BAP, NAA, 2iP, Zeatin are added in appropriate concentrations depending on the experiment.

Selected references

Murashige T and F Skoog (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant*arum 15: 473-497.

Nishizawa S, A Sakai, Y Amano and T Matsuzawa (1993) Cryopreservation of asparagus (*Asparagus officinalis* L.) embryogenic suspension cells and subsequent plant regeneration by vitrification. *Plant Science* 91: 67-73.

Sakai A, S Kobayashi and I Oiyama (1990) Cryopreservation of nucellar cells of navel orange (*Citrus sinensis* Osb. var. *brasiliensis* Tanaka) by vitrification. *Plant Cell Reports* 9: 30-33.

2. Handling of fruits/seeds of non-orthodox species prior to cryopreservation

Rekha Chaudhury, Sangita Bansal and Anang Pal Singh

Introduction

Non-orthodox seeds (comprising intermediate and recalcitrant seeds) are large sized and are shed with high moisture levels (Table 1). They are invariably desiccation- and freeze-intolerant and lose viability after being dried below a critical limit, usually between 12-30% moisture content (MC). At these moisture levels, the seeds cannot be subjected to subzero temperatures since they undergo freeze injury.

Majority of species producing non-orthodox seeds are trees or other plant forms where seed regeneration in the field is highly impractical mainly due to long gestation period. Several tree species and wild species producing even orthodox seeds can be considered for cryostorage in view of apparently there being no need for regeneration if optimal cryoprotocols ensuring longest survival are ensured. There are no defined procedures for conservation of the germplasm of non-orthodox-seeded species. The curator and genebank managers must determine the post-harvest biology and accordingly devise best methodology.

Table 1. Characteristics of orthodox and non-orthodox seeds

S. No.	Characteristics of Orthodox seeds	Characteristics of Non-orthodox seeds
1	Can be dried to 5% or less moisture content (MC)	Can be dried up to critical MC (about 30% in recalcitrant and 10-12% in intermediate seeds)
2	MC homogenous in seed lot	MC vary within seed lot and cotyledon/ embryonic axes (EA)
3	Generally small size	Large fruit and seed size
4	Extended storage life	Short life span
5	Tolerant to desiccation and freezing	Sensitive to desiccation and freezing
6	Annual crops	Perennial trees from moist tropics, temperate & aquatic habitats
7	Maturation drying	No maturation drying
8	Dormant	Metabolically active on shedding
9	Fleshy coverings absent	Seeds covered with fleshy or juicy arilloid and impermeable testa
10	Desiccation sensitivity remains unaltered	Become increasingly desiccation sensitive with storage time

Handling of fruits/seeds of non-orthodox species

Harvest of fruits and seeds

- Fruits/seeds may be collected from trees marked at the time of flowering.
- Harvest the fruits directly from tree avoiding fallen fruits.
- Maturity of fruits may be divided into three developmental stages while keeping optimum gap in days after anthesis (DAA)
    1. Immature- fruits with seeds just after expansion of cotyledons
    2. Partially mature- fruits with seeds having fully expanded cotyledons
    3. Fully mature- fruits with seeds having maximum dry weight
- Seed storage behavior of species, if already known, would help in planning for species-specific collection trips. Information on floral biology, fruit maturity, ecology and seed biology must be gathered for optimizing chances of success.
- Whole fruits of consistent maturity status to be collected from the parent plants to ensure high quality. Preliminary studies would determine which stage of fruit maturity is the best.
- An adequate number of seeds would be required for determining the appropriate protocols for cryopreservation and to utilise in long-term cryostorage. Efforts must be made to collect large fruit quantity. Passport data collection is a very important activity.
- Invariably many of the fruits bearing non-orthodox seeds have pulp and high water content with chances of contamination and hence fruits may be surface decontaminated prior to transport. Fallen (abscised) material showing signs of weathering must be avoided for minimising infections

Post harvest handling and transport

- For effective conservation, rapid transport of whole fruits instead of seeds, seed extraction on the day of experimentation and quick processing for storage within a week of extraction are required. Delay in transport and handling adversely affects the storage behaviour.
- For retention of best seed quality, fruits should be transported to the lab by courier/ speed post and in the best possible conditions, enclosed in moisture-retaining bags or containers.
- Seeds may also be sensitive to chilling and elevated temperatures; hence transport temperature must not be too low or too high.
- The soft and succulent fruits would deteriorate and get infected by the time they are received in the lab even when sent by fastest mode. Hence seeds from half of the fruits may be extracted, coated using bavistin powder, packed in sawdust/ charcoal/ peat moss and should be transported to reach the laboratory within 48 h of extraction.

Laboratory handling

- Fruit characters, as per IPGRI descriptors, like color, firmness, shape, surface features, fresh weight, dimensions and other physical features should be recorded for distinguishing developmental stages and correlating with physiological maturity of fruits and seeds.

- For water content determination, a minimum of ten seeds must be used (on an individual seed/embryo/axis basis/ bulk) and for viability testing 20 to 50 explants may be used.
- The part of the seed most appropriate for conservation must be decided after understanding the morphology and physiology of the whole seed and axes, and ascertaining the ability of explants to regenerate. In large seeded species like coconut and oil palm, excised embryos/ plumules can be taken up and in litchi, jackfruit, *Madhuca* spp. and other similar types, embryonic axes prove to be the explants of choice.
- Seeds should not be retained in fruits for too long as it could lead to vivipary.

Extraction and excision of explants in laboratory

- Seeds should be extracted from fruits using muslin cloth or paper towel (but not washed as moisture would increase) and cleaned thoroughly to remove any fruit part that may cause infection. Extracted seeds should be used up for experimentation within few hours to few days.
- If temporary storage is required, seeds may be stored in sawdust, charcoal or after treating with fungicides like Bavistin or Thiram powder at temperatures between 15 to 20°C. This practice will ensure high survival and pathogen free laboratory storage with retention of initial moisture content.
- In case whole seeds are not being processed, embryos or embryonic axes may be excised from the seeds sterilized under laminar flow cabinet. The exact location of the embryonic axes within the seed should be ascertained (Fig. 1).

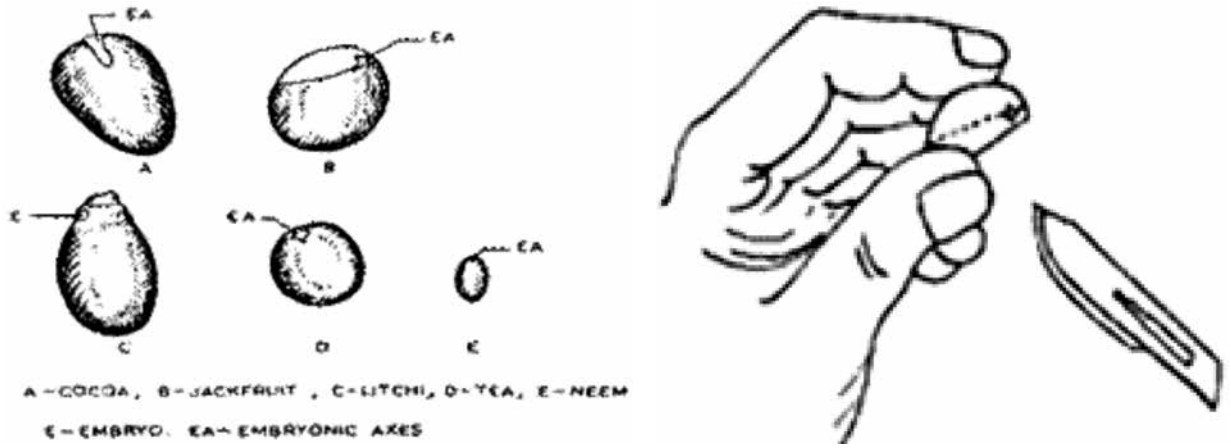


Fig. 1. *Embryonic axes in different seeds and its excision*

- Seeds may be sterilized using sodium hypochlorite (2-2.7%) for 10 min followed by water washings. Embryos and embryonic axes, being deep seated, generally do not carry any infection and hence, 8-10 min treatment with a sterilant is sufficient. The concentration of the disinfectant and the duration of the treatment may be altered as required.
- While excising embryonic axes (EA), the time taken to handle a number of samples should be minimized, so that there are no major differences in moisture contents between axes.

Viability tests

Pertiplate germination

Fresh fruits must be cut open to extract the seeds which are washed thoroughly. The seeds and embryos are placed between two sheets of moistened paper in plastic Petri plates (11 cm diameter) (Fig. 2) and incubated at 27 ± 2°C with 16/8 h light/dark photoperiod. The extrusion of the shoot and/or root indicates germination of the embryo and isolated embryonic axis (Fig. 2).

Fig. 2: *Seed germination on petriplate*

Rolled paper towel (BP method) for germination

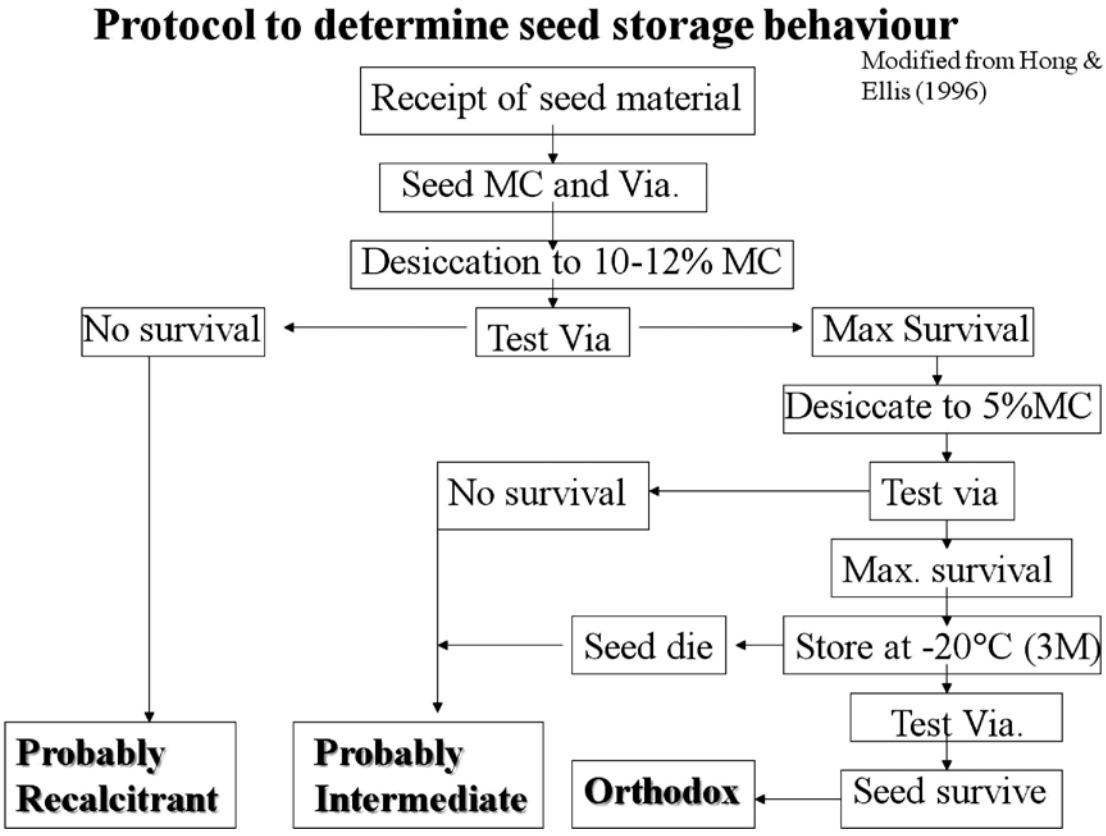
Large seeds are invariably germinated between the paper (BP) using brown germination papers so that the seed surface remains in touch with paper/ water at all times. The seeds are spaced out uniformly between two layers of paper and rolled in towels (Fig. 3). The rolled towels are placed in the germinator in an upright/ lying down position. The germination test is evaluated as normal and abnormal seedlings and dead seeds.

Fig. 3. *Seed germination on rolled paper towel*

In vitro recovery

The embryonic axes, fresh as well as after different treatments, in most cases are cultured on Murashige and Skoog (MS) macro- and micro-nutrients, vitamins, iron, 1gL-1 activated charcoal and 0.17 gL-1 NaH2PO4 supplemented with 1 mgL-1 each of 6-benzylaminopurine (BAP) and α-naphthalene acetic acid (NAA), as defined by Chin *et al.* (1988). Cultures are maintained at 25±2°C with a 16 h photoperiod under a light intensity of 35 μE m-2 s-1. Growth is assessed after 3 weeks of culture and at suitable intervals (Fig. 4), plantlets are subsequently transferred to field. In specific cases other in vitro media can be standardized as per need.

Fig. 4. *In vitro germination of embroyos/embroyonic axes*



Practicals

Extraction of embryos of oilpalm (*Elaeis guineensis*) and *Citrus* species

1. Extract the oilpalm kernels after breaking the hard endocarp of seeds.
2. Soak the seeds for five days in plain water. Change the water daily.
3. Sterilize the intact kernels with 0.2% Bavistin for 10 min.
4. Extract the embryos aseptically using lab grade blade and scalpel.

5. Surface sterilize the extracted embryos with 0.1% mercuric chloride for 5 min and wash thoroughly with sterile distilled water 2-3 times.
6. Determine moisture content gravimetrically by the low constant temperature oven dry method where seeds were dried at 103±2°C for 17 hours (ISTA, 1985).
7. Air desiccate the embryos for 1, 2, 3 hrs. and determine the moisture content at each step.
8. Culture embryos *in vitro* for viability testing by using MS medium in combination with different growth hormones.
9. Extract seeds from Citrus fruits.
10. Briefly wash them in water to remove any adhering pulp.
11. Dry them with blotting sheets.
12. Air dry briefly and carefully remove the seed coat.
13. Determine moisture content gravimetrically by the low constant temperature oven dry method where seeds were dried at 103±2°C for 17 hours (ISTA, 1985).
14. MC is calculated using the formula-

$$\text{Moisture content (\%)} = \frac{\text{B-C}}{\text{B-A}} \times 100$$

Where,
A= Wt. of empty weighing bottle
B= Wt. of bottle + fresh seeds
C= Wt. of bottle + oven dried seeds

MC is expressed on fresh weight basis. Assessments are done on triplicate samples.

S. No.	Air desiccation duration (Hrs)	Moisture Content (%)	Viability (%)	Viability (%) after cryoexposure
1	0			
2	1			
3	2			
4	3			

Extraction of embryonic axes of *Prunus armeniaca* (apricot)

1. Crack open the hard endocarp to extract the seeds.
2. Surface sterilize the seeds with 0.2% Bavistin for 15 min in laminar flow cabinet. Rinse four times with sterile distilled water.
3. Treat with 0.1% mercuric chloride for 5 min and wash thoroughly with sterile distilled water 3-4 times.
4. Aseptically remove brown seed coat and gently separate out the cotyledons snapping one of the attached connections with embryonic axes.
5. Make an incision at the other attached connection of embryonic axes to separate it out.
6. Use 15-20 axes for moisture content determination using low constant temperature oven method.

3. Cryopreservation Using Vitrification Technique

Neelam Sharma, R. Gowthami, Ruchira Pandey and Ramesh Chandra

Introduction

For long term conservation of germplasm cryopreservation is the only method available as of now. It includes freezing of tissue at the temperature of liquid nitrogen (-196°C) or gaseous phase (-140°C) which suspends all metabolic activities. The critical point of all the methods available is to avoid intracellular ice crystal formation. In literature there are 4 types of cryopreservation protocols – (i) Conventional slow freezing; (ii) Simple freezing; (iii) Vitrification and (iv) Desiccation.

Scope of the practical

The scope of present assignment is to demonstrate and provide hands-on experience of cryopreservation of *in vitro* shoot tips using vitrification technique. Vitrification techniques explained herewith are successfully applied for long-term conservation of medicinal plant species.

Vitrification technique

The vitrification technique, developed in 1990s for the cryostorage of cultured plant cells, tissue or organs, is based on the ability of highly concentrated solution of cryoprotectants to supercool to very low temperatures through rapid cooling, to become viscous and solidify without ice crystallization (vitrification). Cryoprotectants (such as PVS2 and PVS3) are used to protect the tissue against the desiccation injury. The most commonly used, highly concentrated cryoprotectant mixture is the plant vitrification solution 2 (PVS2), based on the work of Sakai's group (Sakai *et al.* 1990). Following the dehydration of explants with PVS2, samples are directly immersed in liquid nitrogen to achieve vitrification of intracellular aqueous solution.

Removal of toxic PVS2 (unloading) after rapid thawing is carried out by treatment with highly concentrated sucrose (1.2 M) solution in liquid medium. The method has been successfully utilized for cryopreservation of various *in vitro* cultures; particularly shoot tips of different plants including cherry, taro, banana, lily, apple, pear, etc. Cryopreservation as a means to conserve valuable germplasm of agricultural, horticultural and threatened plant taxa has been reported for over 100 species. However in the literature only limited success has been reported regarding *in vitro* cryopreservation of endangered and medicinal plants.

The present exercise includes various steps of vitrification procedure applied for shoot tip of a threatened medicinal plant, *Bacopa monnieri*.

Equipments and materials

Plant material

In vitro plantlets of *Bacopa monnieri*

Checklist of items required

- Laminar air flow
- Dissecting microscope with light source
- Tissue culture kit (forceps, scalpel, surgical blade etc.)
- Liquid nitrogen in a wide-mouth type Dewar
- Lab top cooler maintained at 0°C or ice box containing crushed ice
- Cryocanes
- Water bath maintained at 42°C
- Polypropylene cryovials (1.0 ml)
- Pasteur pipettes
- Petri dishes (35 mm, 60 mm and 90 mm)
- Cryo vials (2.0 ml)
- Cryovial holder (12 × 12)
- Timers
- Liquid nitrogen
- Aluminum foil to wrap plates
- Sterile filter paper (cut to fit 9 cm Petri dish)
- Parafilm strips
- Safety equipment: gloves and goggles

Solutions/medium (prepared in advance)

- Preculture medium (base medium, no hormones, with 0.3M sucrose and 1% agar) dispensed in petridishes.
- Recovery medium (shoot multiplication medium – MS + 0.2 mg l^{-1} BAP)
- Cryoprotectant solution - Plant Vitrification Solution 2 of Sakai *et al.* 1990 (henceforth referred as PVS2 solution) consisted of 30% (v/v, 3.26 M) glycerol (Sigma-Aldrich®), 15% (w/v, 2.42 M) ethylene glycol (EG) (Sigma-Aldrich®), 15% (v/v, 1.9 M) dimethylsulphoxide (DMSO) (Sigma- Aldrich®), and 0.4 M sucrose dissolved in pH 5.8 liquid MS medium. This mixture is highly viscous and takes some time to completely dissolve in solution. So the constituents were added gradually under slow agitation on a magnetic stirrer for 15 minutes. The solution was autoclaved and dispensed into sterile screw cap bottles (100 ml Schot Duran) and refrigerated.
- Unloading solution - Liquid medium with 1.2 M sucrose

Procedure

A cryopreservation procedure comprises 5 main steps – choice of tissue, chemical pretreatment (dehydration), freezing, thawing and regrowth. Generally rapidly growing tissue with densely cytoplasmic cells gives better results. Hence the shoot tips containing meristem with few leaf primordia are the ideal choice. Chemical treatment involves growing the shoot tips in media with high osmotic followed by treatment with a suitable cryoprotectant. It dehydrates the tissue and lowers its freezing temperature. Fast freezing is achieved by directly plunging the cryovials in liquid nitrogen. Regrowth is accomplished by rapidly thawing the shoot tips by swirling the vial in a warm waterbath (~40°C). This is followed by a step to wash off the cryoprotectant, to reduce the osmotic shock and toxicity associated with cryoprotectant, before transferring the shoot tips to recovery media for regrowth. Successive steps of the technique are listed below:

Preparation of plant material

Four-month-old shoot cultures, on shoot multiplication medium, were the source of shoot tips.

Excision and pre-culturing of shoot tips (Day 1 and 2) (Fig. 1)

- Preparation of preculture medium petriplates: Pour 2 ml of preculture medium in 60 mm petri dish and leave it for solidification
- Dissection of shoot tips:

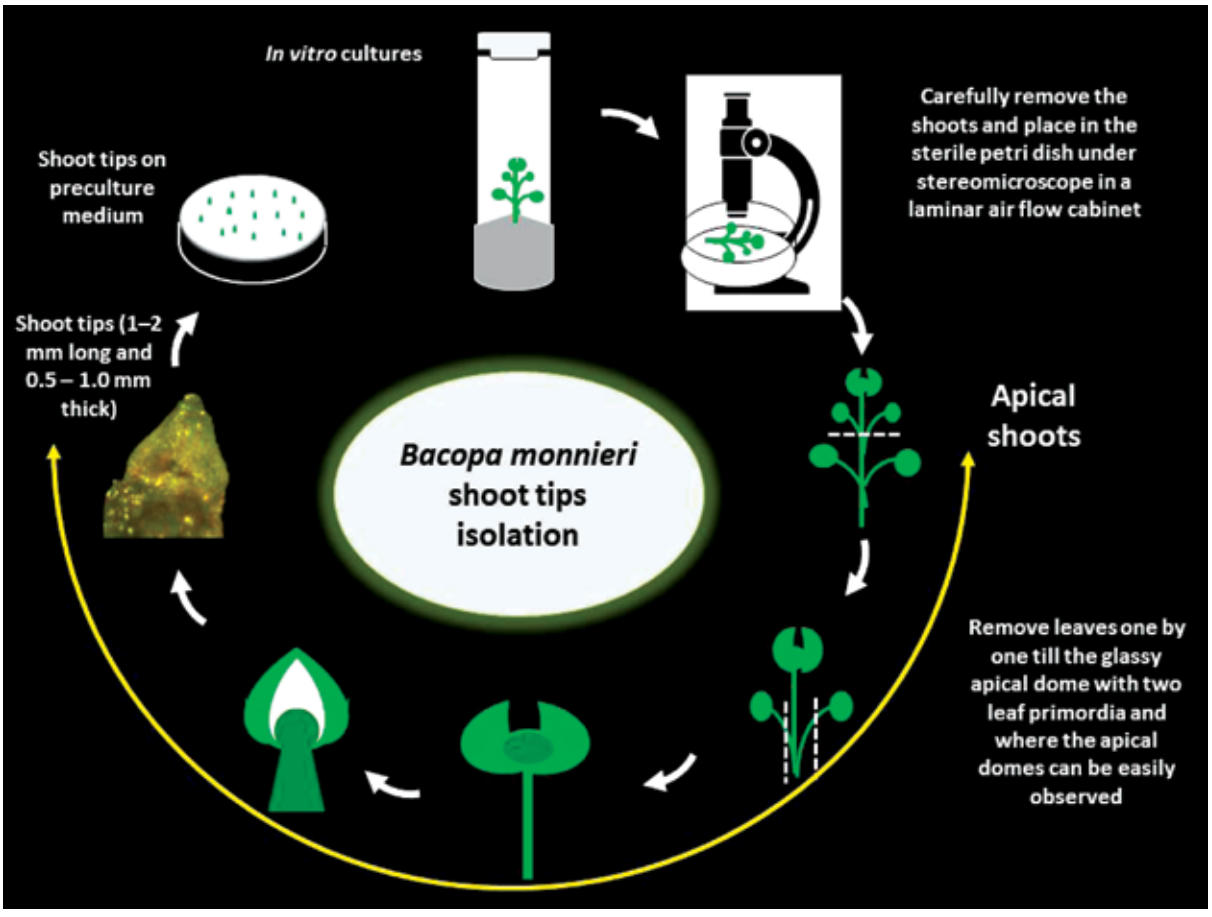


Fig. 1. *Stepwise procedure for shoot tip isolation in B. monnieri*

- Select healthy proliferating *in vitro* cultures grown on shoot multiplication medium for four months
- Carefully remove the shoots and place in the sterile petri dish under stereomicroscope in a laminar air flow cabinet
- It has been observed that shoot tips of *B. monnieri* were covered by overlapping leaves. Excise shoot tips using a fine forceps and sharp surgical blade.
- Remove leaves one by one till the glassy apical dome with two leaf primordia and where the apical domes can be easily observed
- Cut the base of the shoot tips (1–2 mm long and 0.5 – 1.0 mm thick) and place in a preculture medium (20-25 shoot tips in each petriplate)
- Seal the Petri dish with Parafilm and incubate for two days at 22°C

Day 3: Cryopreservation

Dehydration with PVS2

- Label cryovials and place in a labtop cooler in a laminar air flow
- Unscrew lids but leave them on top of vials. Add 1 ml of pre-chilled PVS2 to the cryovials using a sterile Pasteur pipette. To keep the pipette sterile, place sterile pipette tip first in a sterile petri dish, with the lid covering much of the pipette
- Using a forceps add pretreated meristems to the vials, 10 per vials
- Incubate for 35 min at 0°C

Rewarming/ Thawing

To avoid detrimental recrystallization during thawing, rapid thawing is recommended. Thawing of the tissues at around 40°C is essential. The next step therefore, is to place cryovials with frozen shoot tips in a water bath immediately after removing from LN.

- Fill a plastic beaker with hot water and maintain the temperature of 42°C and one beaker with water at 25°C or set water bath at required temperatures
- Remove the cryovials from LN and quickly place it in a 42°C water and rinse it for 2 min.
- Immediately after 2 min., transfer cryovials to beaker with cold water (25°C) and rinse it for 1 min
- Swab the adhered water to the cryovial using cotton (to avoid contamination)

Unloading

- Pour unloading solution in petridish (60 mm)
- Place vials in the labtop cooler after shaking off excess water
- Place vials in the hood and loosen the caps
- Drain off cryoprotectant down to 0.25 ml (where the tube tapers) and immediately add pre-chilled liquid MS medium supplemented with 1.2 M sucrose to the top of the tube. For controls, this step is done after desired PVS2 dehydration
- After two quick washes with unloading solution, add 1 ml of US and incubate for 20 min

Recovery and regrowth

- After removal of PVS2, transfer the shoot tips on to a two layers of sterile filter paper discs in a petri dish
- Transfer the controls [precultured (without PVS2 dehydration) and after PVS2 dehydration but non-frozen] onto regeneration medium (MS + 0.2 mg l^{-1} BAP)
- For recovery growth of the thawed shoot tips, place the shoot tips on the recovery medium and keep it in dark for 4 days at ~25°C (wrapped with aluminum foil to provide dark condition)
- After 4 days, remove aluminum foil and incubate at 25–26°C under light from cool white fluorescent lamps (50–80 μmol m^2 s^{-1}; 16 h light: 8 h dark) (culture room conditions)
- Check for regrowth weekly and record survival (%) and plantlet growth (%), after 4 - 6 weeks. Survival is assessed as the ability of shoot tips to turn green. Regrowth is recorded as the percentage of total number of shoot tips cultured that formed normal shoots after plating
- Transfer the recovered shoots to test tubes with regrowth medium

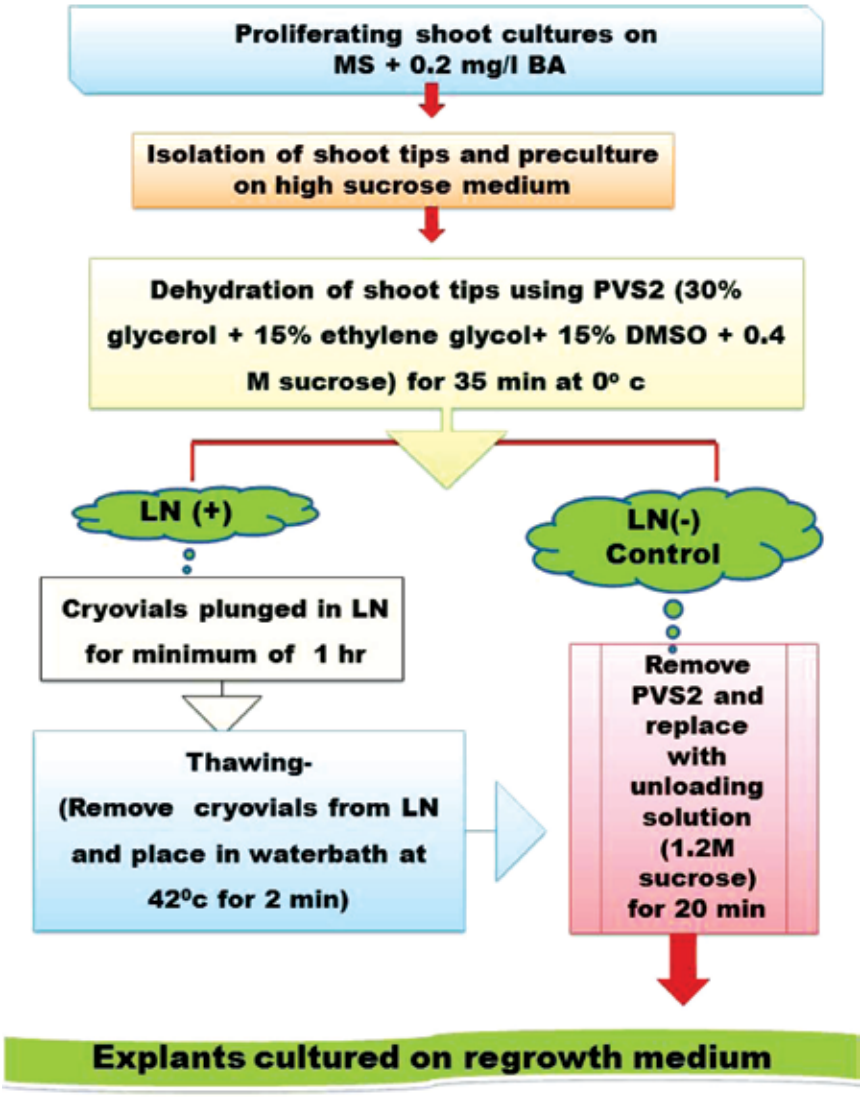


Fig. 2. *Flow chart of cryopreservation of Bacopa monnieri using vitrification*

Selected references

Reed BM (2008) *Plant Cryopreservation- A Practical Guide*. Springer Publication, New York, pp 511.

Sakai A, S Kobayashi and I Oiyama (1990) Cryopreservation of nucellar cells of navel orange *(Citrus sinensis* Osb. var. *brasiliensis* Tanaka) by vitrification. *Plant Cell Rep.* 9: 30-33.

Sharma N and B Sharma (2003) Cryopreservation of shoot tips of *Picrorrhiza kurroa* Royle ex Benth., an indigenous endangered medicinal plant through vitrification. *CryoLetters* 24: 181-190.

Sharma N, R Satsangi and R Pandey (2009) Cryopreservation of shoot tips of *Bacopa monnieri* (L.) Wettst. – A Commercially important Medicinal Plant, by vitrification technique. In: Abstr., 1st International Symposium on *Cryopreservation in Horticultural Species* at Leuven, Belgium, April 5-8 2009, p 125.

Sharma N, R Satsangi and R Pandey (2010) Cryopreservation of shoot tips of *Bacopa monnieri* (L.) Wettst by vitrification technique. *Acta Hort.* 908: 283-288.

Sharma N, R Singh, R Pandey and N Kaushik (2017) Genetic and biochemical stability assessment of plants regenerated from cryopreserved shoot tips of a commercially valuable medicinal herb *Bacopa monnieri* (L.) Wettst. *In Vitro Cell Dev Biol—Plant* 53: 346-351 DOI 10.1007/s11627-017-9826-5.

Sharma N, S Vimala Devi and R Satsangi (2007) Biotechnological approaches in multiplication and conservation of plants of medicinal value. In: PK Shukla and OP Chaubey (eds) *Threatened Wild Medicinal Plants: Assessment, Conservation and Management.* Anmol Publications Pvt. Ltd., New Delhi, India, pp. 248-257.

4. Cryopreservation of shoot apices using V cryo-plate and D cryo-plate method

T. Niino, Sandhya Gupta and D.P.S. Meena

Introduction

Yamamoto *et al.* (2012) developed an effective cryopreservation protocol using aluminium cryo-plates (V cryo-plate method) for *in vitro*-grown shoot tips of mulberries (*Morus* spp.) originated from the tropics and subtropics. This method has two main advantages: 1) it is a user-friendly procedure; and 2) it guarantees higher cooling and warming rates of treated explants. Protocols using V cryo-plate method have been reported recently for strawberry, Dalmatian chrysanthemum, mint, mulberry, carnation and mat rush shoot tips/ buds. A cryopreservation protocol based on air dehydration of explants placed on aluminium cryo-plates, termed D cryo-plate was successfully developed for *in vitro* mat rush (*Juncus decipiens* Nakai) lateral buds (Niino *et al.,* 2014). In the D cryo-plate method, shoot tips/buds attached to the cryo-plates are dehydrated under the laminar air flow cabinet after loading treatment with 2 M glycerol and adequate sucrose solution for inducing tolerance to dehydration (osmoprotection). D-cryoplate method is more advantageous than the other method: 1) it is a user-friendly; 2) PVS2 solution, may be toxic for some species, is not used; and 3) It also provides higher cooling and warming rates of treated explants.

Stock cultures

The nodal segments with a lateral bud (about 5 mm) from the shoot cut and plated on 20 ml solid MS medium in Petri dishes (about 90 × 20 mm) and cultured for about 2 weeks under standard conditions. One Petri dish containing about 40 nodal segments would be used for excising shoot tips emerging from the nodal segments.

Plant material

Shoot apices excised from *in vitro* cultures of *Fragaria* and/or carnation

Tools

- Petri dish
- Cryo-plate No.2 and No.3
- Micropipettes (50-100 μl and 1 ml) and their tips
- Filter papers or replacement

- Pipetting reservoirs or Petri dishes
- Cryo-canes
- Cryotubes and stand
- Timer
- LN and Dewar flask
- Culture media

Media/ Solutions

Experiment 1: V cryo-plate method

- Shoot tips: at least 5 shoot tips (1.5 mm length) on MS medium with 0.3 M sucrose
- 2% Na-alginate solution with 0.4 M sucrose
- 0.1 M $CaCl_2$ solution with 0.4 M sucrose
- LS solution (2M Glycerol + 1.4 M sucrose)
- PVS2 solution1.0 M sucrose solution

Experiment 2: D cryo-plate method

- Shoot tips: at least 5 shoot tips on MS medium with 0.3 M sucrose
- 2% Na-alginate solution with 0.4 M sucrose
- 0.1M $CaCl_2$ solution with 0.4 M sucrose
- LS solution (2M Glycerol + 1.4 M sucrose)
- Petri dish with 35g silica gel and a filter paper
- 1.0 M sucrose solution

V cryo-plate method (Yamamoto et al., 2012 a, b)

The successive steps of the V cryo-plate procedure are given hereunder (Photos © T. Niino).

Step 1. Adhesion of shoot tips on the cryo-plates (Fig.1)

1. Set the cryo-plates (mainly No.2) on the Petri dish (Fig. 1A). At one time, treatment of 2–6 plates is preferable.
2. Place sodium alginate solution (2–2.5 μL) on the wells of the aluminium plate using a micropipette (Fig. 1B, C).
   - ♦ Wipe cryo-plates by paper soaked with ethanol, if the droplet of the alginate solution does not make sphere on the well.
   - ♦ If the shoot tips are large, the amount of sodium alginate solution should be increased or use larger well plate.
   - ♦ Sodium alginate solution including 0.4 M sucrose could also be used.
3. Place the precultured shoot tips in the wells one by one with the tip of a scalpel blade and slightly press the shoot tips to make them fit in the plate's wells (Fig. 1D).

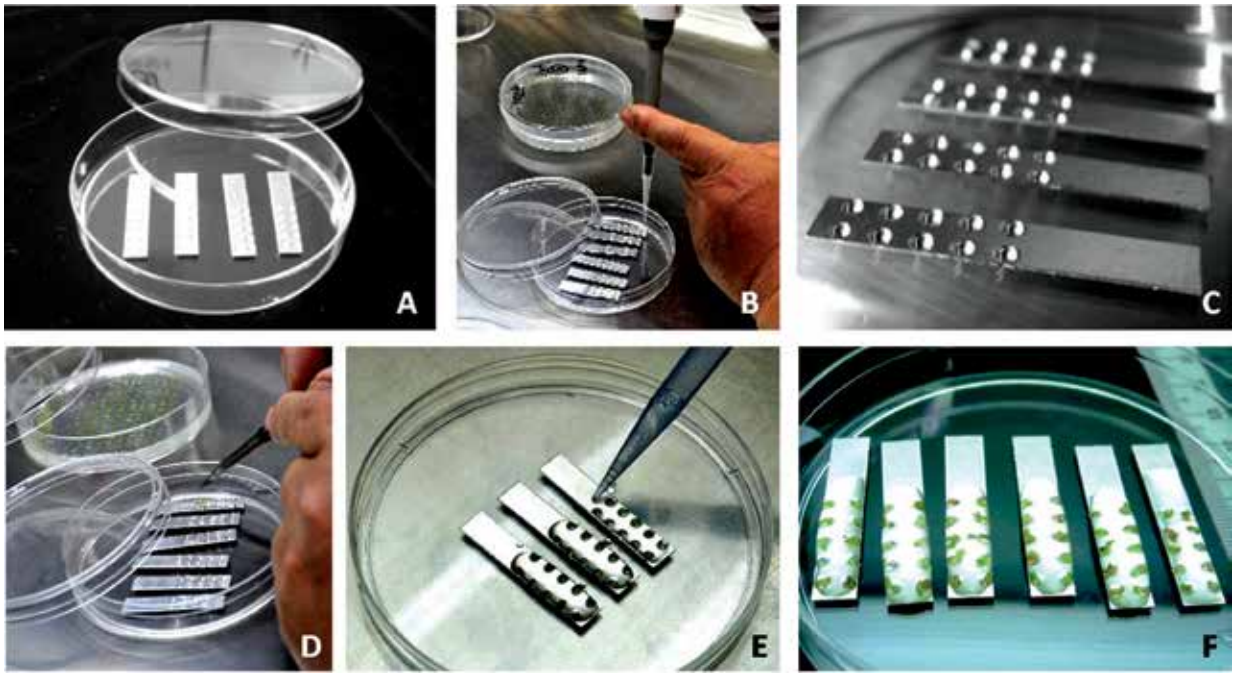


Fig. 1. *Adhesion of shoot tips on the cryo-plates*

- If the shoot tips are large and not covered, add more alginate solution over the shoot tips in order to cover them completely.

4. Pour the calcium chloride solution dropwise on the section of the aluminium plate where the shoot tips are located until they are covered and wait for about 15 min to achieve complete polymerization (Fig. 1E).
   - The duration of polymerization is around 15 min at room temperature.
   - The tip of micropipette should not touch the shoot tips and the alginate solution.
   - The calcium chloride solution should be placed gently over the plate, the solution would cover the cryo-plate by surface tension.
5. Remove the calcium solution from the cryo-plate by sucking it gently with a micropipette.
   - The tip of micropipette should not touch the shoot tips and gel.
   - The calcium chloride solution should be sucked gently with a micropipette.
   - The plate is tapped on sterile filter paper (kept in another Patri plate) so that the extra solution over the plate should be soaked into the filter paper before the LS treatment.

Step 2. Treatment by LS and PVS2 (Fig. 2)

1. Place the cryo-plate with attached shoot tips in a 25 mL pipetting reservoir (NSG Precision Co. Mie, Japan) filled with 20 mL loading solution (LS). Shoot tips are osmoprotected at 25°C for adequate time. The LS solution contains 2 M glycerol + various concentrations of sucrose in liquid MS basal medium (Fig. 2A).
   - The concentrations of sucrose are from 0.4 M to 1.6 M usually.
   - Small bottles, Petri dishes and cryotubes may be used in place of pipetting reservoir.

Fig. 2. *Treatment by LS and PVS2*

2. Remove the cryo-plate from LS and place it in a 25 mL pipetting reservoir filled with PVS2 (about 20 mL). Shoot tips are dehydrated at 25°C for various durations (Fig. 2A, B).
   - ♦ The duration of dehydration by PVS2 is important.
   - ♦ PVS3 or other PVSs can be used for dehydration treatment.
   - ♦ Before immersion in PVS2, the plate should be wiped on the edge of the reservoir.
3. After dehydration, the cryo-plates are removed from the reservoirs with PVS2 and the PVS2 drops are removed with filter paper (Fig. 2C). The cryo-plates are transferred to uncapped 2 mL cryotubes, which are held on cryo-canes (Fig. 2D), and directly plunged in LN where they remained for at least 30 min (Fig. 2E).
   - ♦ When the cryotube is held on cryo-cane, cryotube should be slanted a little to make handling easy.

Step 3. Regrowth (Fig. 3)

1. Cryotubes are retrieved from LN (Fig. 3A) and the cryo-plates with shoot tips are transferred from the cryotubes and immersed in 2 mL 1 M sucrose solution in MS basal medium contained in another 2 mL cryotube (Fig. 3B). Shoot tips are incubated in this solution for 15 min at room temperature (Fig. 3C).
2. Shoot tips with or without alginate gel are transferred onto solid MS medium (Fig. 3D, E). Post-rewarming regrowth (regrowth level) is evaluated after 2–4 weeks of culture at 25°C under standard conditions by counting the number of explants that develop normal shoots (Fig. 3E).

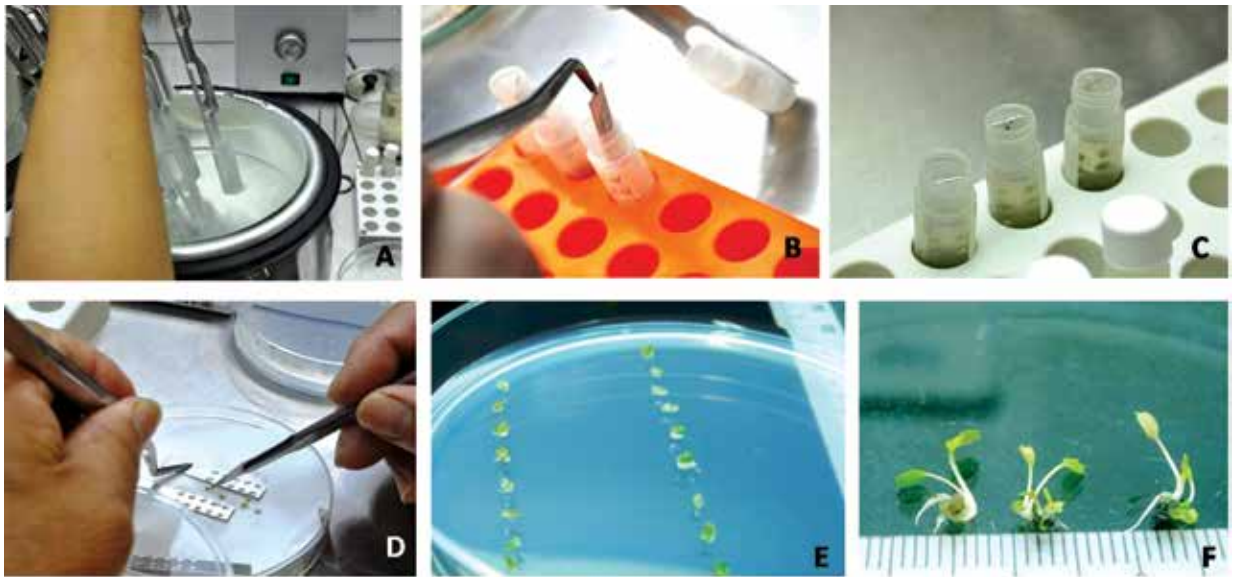


Fig. 3. *Regrowth*

D Cryo-Plate method (Niino *et al.*, 2013, 2014)

Some plants displayed low regrowth after cryopreservation due to their sensitivity to treatment with the PVS2 solution. Avoiding the damage associated with sensitivity of plant material to plant vitrification solution, an air dehydration method was effective. So, air dehydration method using the cryo-plates (D cryo-plate method) was developed. This D cryo-plate is a method that combines encapsulation-dehydration and V cryo-plate (Niino *et al.*, 2014). Tools needed to perform this method are almost the same as indicated in V cryo-plate method.

The successive steps of the D cryo-plate procedure are given hereunder (Photos© T. Niino).

Step 1. Adhesion of shoot tips on the cryo-plates (Fig. 4)

1. Set the cryo-plates (mainly No. 3) on the Petri dish, and place sodium alginate solution (about 4 μL) on the wells of the aluminium plate using a micropipette (Fig. 4A).
   - ♦ Sodium alginate solution including sucrose and/or glycerol could also be used.
   - ♦ A volume of sodium alginate solution is different by well size of cryo-plate and specimens size used.

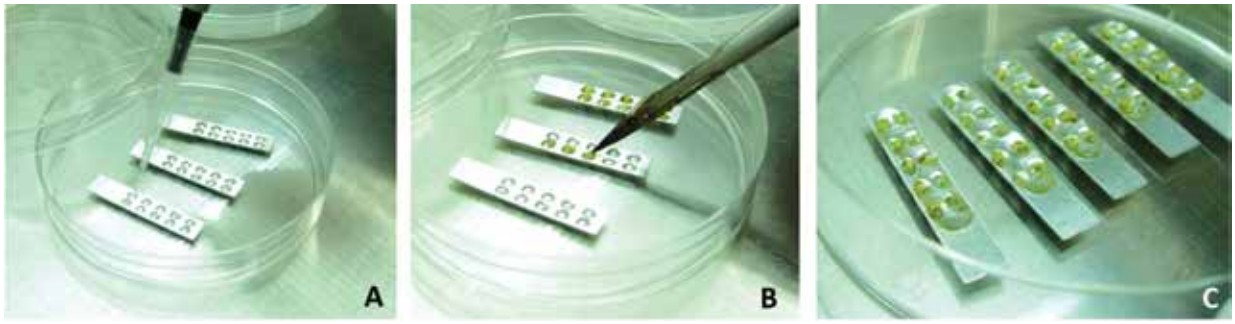


Fig. 4. *Adhesion of shoot tips on the cryo-plates*

2. Place the precultured shoot tips in the wells one by one with the tip of a scalpel blade and slightly press the shoot tips to make them fit in the plate's wells, and add more alginate solution over the shoot tips to cover them completely (Fig. 4B).
3. Pour the calcium chloride solution dropwise on the section of the aluminium plate where the shoot tips are located until they are covered and wait for about 15 min to achieve complete polymerization (Fig. 4C).
    - ♦ The tip of micropipette should not touch the shoot tips and the alginate solution.
    - ♦ The calcium chloride solution should be placed gently over the plate, the solution should cover the shoot tips by surface tension.
    - ♦ When sodium alginate solution is included with sucrose and/or glycerol, calcium chloride solution should be included sucrose and/or glycerol.

Step 2. Treatment by LS and desiccation by airflow (Fig. 5)

1. Removing the calcium solution from the cryo-plate, place the cryo-plate with attached shoot tips in a 25 mL pipetting reservoir filled with a 20 mL loading solution (LS). Shoot tips are osmoprotected at 25°C for adequate time. The LS solution contains appropriate concentrations of glycerol (usual 2 M) and sucrose in liquid MS basal medium (Fig. 5A).
2. Remove the cryo-plate from the LS and place it on paper filter in Petri dish. Then desiccate the cryo-plates in laminar flow cabinet for an adequate time at 25°C, 40– 50% RH (Fig. 5B).
    - ♦ Before air desiccation, the LS drops are removed with filter papers.
    - ♦ Optimal desiccation time would be change by the capacity of laminar flow cabinet and humidity of room.
    - ♦ The silica gel in Petri dish can be used for desiccation, too.

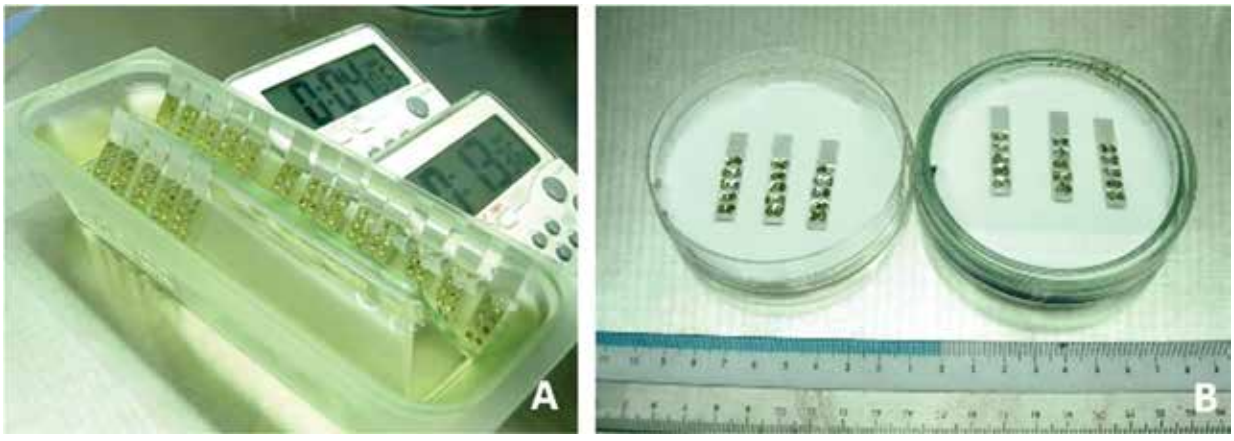


Fig. 4. *Treatment by LS and desiccation by airflow*

3. After dehydration, the cryo-plates are transferred to uncapped 2 mL cryotubes, which are held on cryo-canes, and directly plunged in LN where they remained for at least 30 min.
4. Regrowth and long-term storage in the liquid phase of the LN tank procedure are the same as indicated in V cryo-plate method.

References

Niino T, S Yamamoto, K Fukui, CR Castillo Martínez, M Valle Arizaga, T Matsumoto, and F Engelmann (2013) Dehydration improves cryopreservation of mat rush (*Juncus decipiens* Nakai) basal stem buds on cryo-plates. *Cryo Lett* 34: 549–560

Niino T, Wunna, K Watanabe, N Nohara, T Rafique, S Yamamoto, K Fukui, M Valle Arizaga, CR Castillo Martinez, T Matsumoto and F Engelmann (2014) Cryopreservation of mat rush lateral buds by air dehydration using aluminium cryo-plate. *Plant Biotechnology* 31, 281–287 (2014) DOI: 10.5511/plantbiotechnology.14.0624a

Yamamoto S, T Rafique, K Fukui, K Sekizawa, A Koyama, T Ichihashi and T Niino (2012a) Development of an effective cryopreservation protocol using aluminium cryo-plates for in vitro-grown shoot tips of mulberries (*Morus* spp.) originated from the tropics and subtropics. *Sanshi-Konchu Biotechnol.* 81: 57–62 (in Japanese with English summary)

Yamamoto S, T Rafique, K Fukui, K Sekizawa and T Niino (2012b) V-Cryo-plate procedure as an effective protocol for cryobanks— Case study of mint cryopreservation. *Cryo Lett* 33: 12–23

5. Cryopreservation of budwoods

Vartika Srivastava, Sangita Bansal and D.K. Nerwal

Introduction

Vegetative dormant buds are quite commonly found in woody tree species. The buds bear a growing meristematic dome surrounded with many layers of scales. Under certain environmental conditions, such as extreme heat, drought or cold, these buds enter into a suspended growth phase, where most of the physiological activities reduce to a minimal level, analogous to a seed. The only difference is that the moisture content of these buds is not as low as that of seeds. Throughout the world, dormant buds have been identified as one of the best vegetative propagules for long-term conservation of woody crop species due to the ease and low cost involved. Since centuries these vegetative dormant buds are utilized for propagation of identical clones in most of the fruit crops either through cutting, budding, layering and grafting etc. and hence could be ideal for long term conservation of selected woody crops.

For dormant bud cryobanking, the bud sticks are first dehydrated to obtain optimum moisture content followed by step wise freezing. Buds can be conserved for long-term, utilizing any technique of cryopreservation depending upon the regeneration procedure standardized. For species in which the *in vitro* regeneration protocol is available, the excised bud can be cryopreserved through vitrification, encapsulation as well as step wise freezing and regenerated *in vitro*. Cryopreservation using dormant buds as explants costs less as compared to the extensive procedures of conserving shoot tips derived from aseptic cultures.

Cryopreservation of vegetative propagules including dormant buds in case of woody plants was first reported by Sakai (1960). This technique is being profusely utilized in case of conservation of fruit trees, *viz*., *Malus* spp. (Forsline *et al*., 1998; Sakai *et al*., 1978; Tyler *et al*., 1988), *Pyrus* (Suzuki *et al*., 1997), *Morus* (Niino *et al*., 1993) etc. Cryopreservation of dormant buds offers distinct advantages, *viz*., maintenance of genetic integrity, long-term conservation, simple process, space efficient and low cost of maintenance.

Advantages of dormant bud cryobanking

- Budwood conservation offers advantage of clonal integrity. Vegetative buds in their dormant state are one of the best propagules to conserve and propagate identical clones of fruit crops and widely used as a routine procedure for cryopreservation of *Malus* spp., *Morus* spp., *Salix* spp. etc.
- Dormant buds can be transported easily and regenerated via grafting or budding on the suitable root stocks.

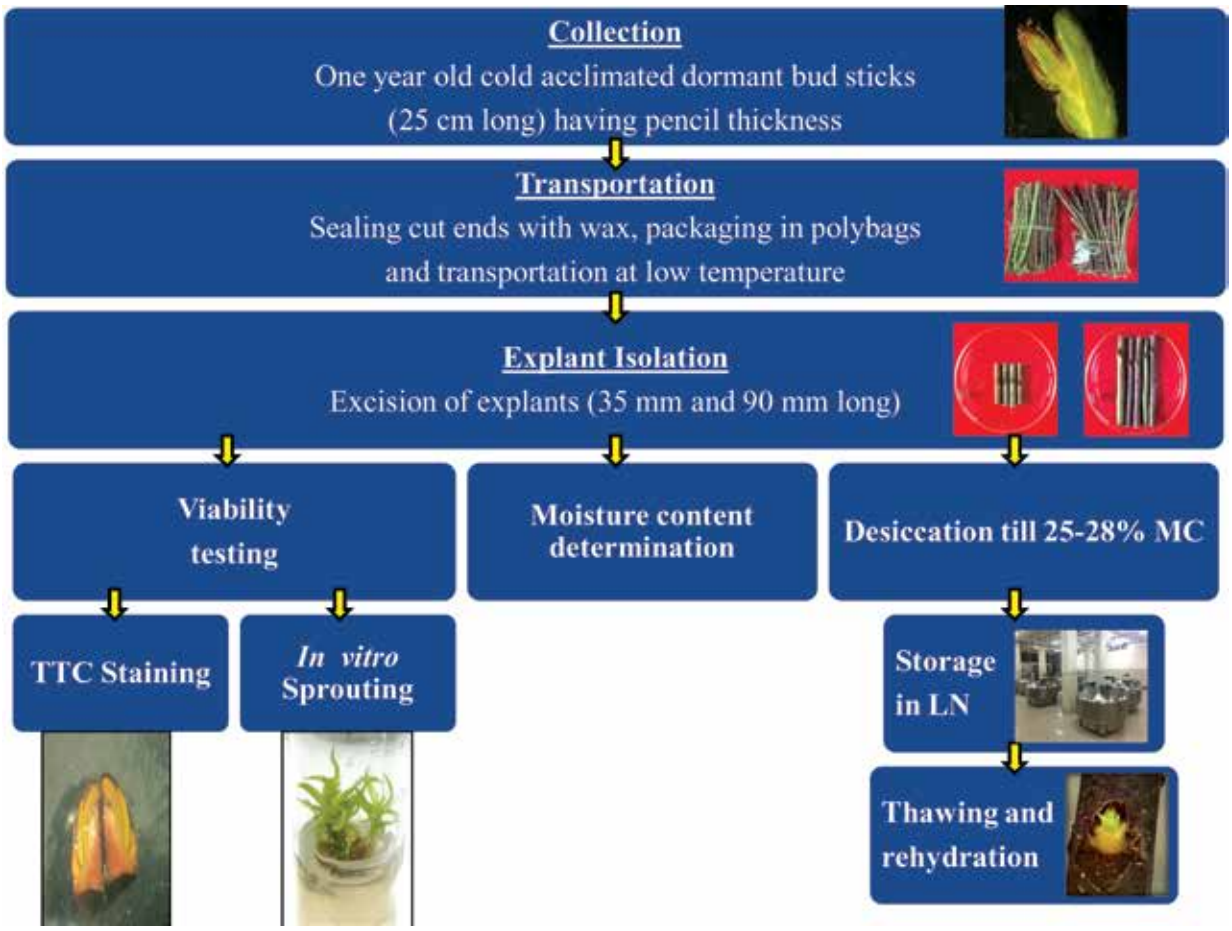


Fig. 1. *Basic steps in cryopreservation of dormant buds*

- As compared to *in vitro* cryopreservation, this method is relatively simple and space efficient.
- Dormant buds are amenable to long term conservation.
- It offers a costs effective technology when compared to new cryopreservation techniques.

Budwood cryopreservation protocol

Collection and transportation of budwood

Completely cold acclimated dormant bud sticks of temperate woody tree species (e.g. almond and walnut) are collected during peak winter months when leaves of the trees are shed and buds are totally compacted. Preferably twigs are collected after trees have remained constantly under snow for 5-7 days. For collection of buds, 25 cm long twigs of last one year's growth of pencil thickness; having dormant buds are harvested from plants growing at the field genebank in winter seasons from December to February. To retain the moisture within the twigs, the cut ends are sealed with wax after severing from the tree and then packed.

Storage and handling in laboratory

On receipt the twigs are wiped with ethanol (70%) in a cool room, wrapped in plastic bags and stored in refrigerator at 4-5°C temperature. Dormant buds are extracted initially under the stereo microscope for morphological studies.

Processing of explants prior to cryopreservation

Nodal sections (3.5 cm) and sticks (9 cm long) of pencil thickness with vegetative buds are cut from the well acclimated twigs/scion. Viability of cleaned and descaled fresh buds is checked through TTC as well as *in vitro* regeneration method. Viability through TTC (2%) is checked by putting pieces of buds in the TTC solution kept in dark overnight. *In vitro* regeneration is checked via inoculating surface sterilized buds (washed with Tween 20 for 15 min followed by washing with running tap water and treatment with 0.1% $HgCl_2$ for 8-9 min followed by 3 washings with autoclaved distilled water) on suitable culture medium.

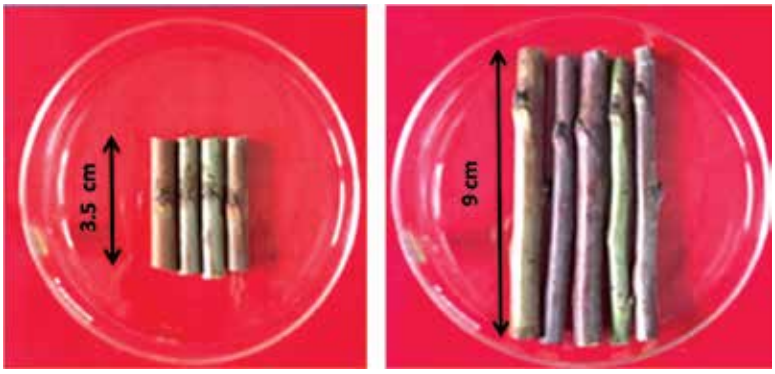


Fig. 2. *Explants for cryopreservation*

Moisture content determination and air desiccation

Moisture content (MC) of fresh and desiccated buds is estimated using low constant temperature oven method. About 5-6 buds are cut to small pieces and used for MC determination by drying at 103°C ± 2°C temperature in an oven for 17 h. For processing the excised buds for desiccation, these are kept in a muslin cloth placed inside silica gel in desiccators of about 4 litre capacity maintained at 4°C. Slow desiccation of nodal sections and twigs is done by placing the explants in wire drying rack or meshes and kept exposed to low temperatures in refrigerator. Depending on the initial moisture contents, the target moisture contents between 25 to 28% may be achieved by desiccating the chipped buds for 2 to 3 hrs and nodal sections for 18-20 hrs in silica. Buds processing is carried out under non-sterile conditions, only those buds which need to be regenerated *in vitro* after cryopreservation and thawing are sterilized as per standard procedure under laminar air flow.

Storage in LN

(A) Programmable freezing

A programmable freezer has a freezing chamber cooled by LN supplied from a pressurized LN vessel. A freezing rate of 1°C per minute is given from 5°C to the terminal prefreezing temperature of -30°C. Samples are held at this temperature for 30 min followed by a rapid immersion in LN. Although, a programmable freezer is quite expensive and may not be available in all the labs, so a simpler step wise freezing method can be adopted.

(B) Step-wise freezing

Dormant buds (nodal sections as well as sticks), pre-desiccated to optimal moisture contents are subjected to freezing after enclosing in 50 ml cryovials. Slow step wise freezing is achieved by sequentially lowering the temperature at -5°C/day using deep freezers up to terminal temperature of -25°C. For this, the cryovials/ polyolefin tubings enclosing the explants are shifted sequentially at 5°C, -5°C, -10°C, -15°C, -20°C and -25°C, keeping at each of the temperatures for a minimum of 24h. Samples are held at terminal temperature of -25°C for 24-48 h followed by a rapid immersion in LN. Simple fast freezing may be achieved by direct

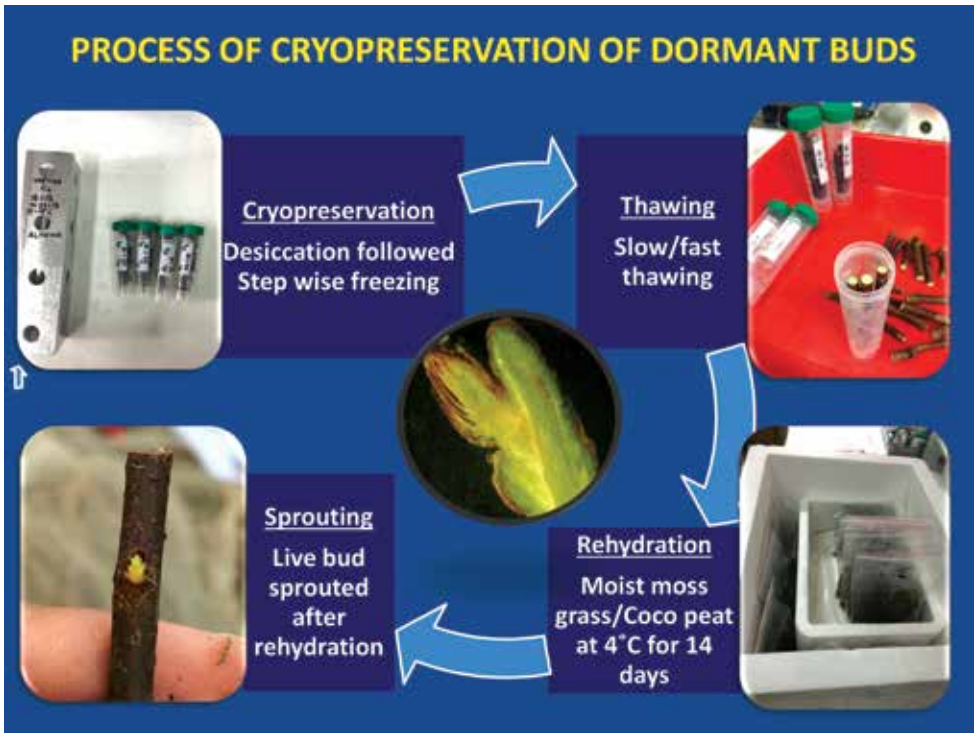


Fig. 3. *Dormant bud cryopreservation protocol*

plunging of pre-desiccated buds in liquid nitrogen without any pre-freezing. The excised buds may also be processed and cryostored as above and must be enclosed in cryovials (50 ml capacity).

Thawing and rehydration of buds

Thawing is the most critical step of recovery in dormant bud cryopreservation. The protocol of thawing must be standardized for each species. Basically two types of thawing are followed i.e., slow thawing and rapid thawing. In slow thawing, the cryovials are simply removed from LN storage tank and placed in a refrigerator (4°C) for 24 hours before subjecting to rehydration while in rapid thawing, the cryovials after removal from LN, are suspended in lukewarm water (temperature at 37°C) for about 20-30 minutes followed by rehydration.

Recovery through *in vitro* and *in vivo* methods

Recovery of the thawed buds is done via *in vitro* regeneration as well as *in vivo* budding. But before that, the buds/nodal sections are rehydrated in moist peat moss for

Table 1. Dormant bud conservation in different Genebanks of the world

Plant material	Genebank/country	Reference
Mulberry and *Malus* sp. (387 accessions)	National Bureau of Plant Genetic Resources (NBPGR), New Delhi, India	ICAR-NBPGR Annual Report (2017-18)
Apple (2200 accessions)	National Center for Genetic Resources (CNGR), Fort Collins, USA	Forsline *et al.* (1999)
Mulberry (420 accessions)	National Institute of Agrobiological Resources (NIAR), Yamagata, Japan	Niino (1995)

10-15 days at 4°C temperature (in case of temperate species). For *in vivo* recovery, chip of bud isolated from the rehydrated nodal sections is budded to a suitable rootstock in field and bud emergence and re-growth is recorded. In case of test of *in vitro* recovery of buds, the buds are surface sterilized followed by removal of 4-7 outer scales and culturing on suitable regeneration medium.

Selected references

Forsline PL, LE Towill, JW Waddell, C Stushnoff, WF Lamboy and JR McFerson (1998) Recovery and longevity of cryopreserved dormant apple buds. *J. Amer. Soc. Hort. Sci.* 123(3): 365-370

Forsline PL, JR McFerson, WF Lamboy and Towill LE (1999) Development of base and active collections of Malus germplasm with cryopreserved dormant buds. *Acta Hort.* 484: 75–78.

ICAR-NBPGR (2018) Annual Report 2017-2018, ICAR- National Bureau of Plant Genetic Resources, New Delhi, India. 181 p.

Niino T (1995) Cryopreservation of Germplasm of Mulberry. *In:* Biotechnology in Agriculture and Forestry, Volume 32, Cryopreservation of Plant Germplasm I; Bajaj YPS, Ed.; Springer Verlag: Berlin, Germany, pp. 102–113.

Niino T, A Koyama, K Shirata, S Ohuchi, M Suzuki and A Sakai (1993) Long term storage of mulberry winter buds by cryopreservation. *J. Seric. Sci. Jpn*. 62: 431-434.

Sakai A (1960). Survival of the twigs of woody plants at -196C. *Nature*. 185: 393-394.

Sakai A and Y Nishiyama (1978) Cryopreservation of winter vegetative buds of hardy fruit trees in liquid nitrogen. HortScience. 13: 225-227.

Suzuki M, T Niino, T Akihama and S Oka (1997) Shoot formation and plant regeneration of vegetative pear buds cryopreserved at-150 C. *J. Jap. Soc. Hortic. Sci.* 66(1): 29-34.

Tyler NJ, C Stushnoff and LV Gusta (1988) Freezing of water in dormant vegetative apple buds in relation to cryopreservation. *Plant Physiol*. 87: 201-205.

6. Cryopreservation of non-orthodox seeds using varied techniques

Hugh Pritchard, Rekha Chaudhury and Anang Pal Singh

Introduction

Difficult-to-store non-orthodox (intermediate and recalcitrant) seeds are abundantly found growing in moist ecosystem of tropics, subtropics and in aquatic and riparian environments. Seeds are subjected to high humidity during seed development, maturation and at harvest. Due to these conditions seeds do not undergo maturation drying as a final, preshedding development phase. Short-, medium- and long-term seed storage can only be recommended when seed storage behaviour and developmental stage when the seeds exhibit tolerance to desiccation is known. Recalcitrant seeds at various developmental stages are reported to have varying degree of tolerance to desiccation. This also varies among species (Hong and Ellis, 1996), for example, immature/partially mature embryos of jackfruit and litchi have been found to be more adaptable to manipulation than mature embryos/embryonic axes (Chaudhury *et al.,* 2011). Gradual increase and decrease in desiccation tolerance with development and with initiation of germination have been observed in both non-orthodox and orthodox species. Rapid and careful handling, vitrification and use of embryonic axis have been found effective in cryopreservation for tea, black pepper, cardamom, almond, citrus, trifoliate orange, neem and other tropical fruit species at NBPGR.

The non-orthodox seeded species can be designated as highly recalcitrant (e.g. mango, litchi, jackfruit, mahua, jamun, mangosteen etc.), moderately recalcitrant (papaya, several citrus species, black pepper, banana etc.) and weakly recalcitrant (some citrus species, bael, custard apple etc.). This classification is based on the degree of desiccation sensitivity, hydrated storage life span and chilling sensitivity. Cryopreservation of desiccation-sensitive tissues, like that of non-orthodox seeds are only possible once the moisture content of explants is reduced to an optimal level with reasonably high viability and is able to survive freezing stresses as low as -196°C.

Various desiccation techniques for seeds and embryonic axes, namely air desiccation, pregrowth-desiccation, vitrification and encapsulation-dehydration are in use at cryolab of NBPGR (Malik *et al.,* 2012). Cryoprotocols of vitrification and encapsulation have been successfully attempted in embryonic axes of *Artocarpus heterophyllus, Litchi chinensis*, *Poncirus trifoliata* and *Citrus* spp. (Malik and Chaudhury, 2006).

Cryopreservation of desiccation-sensitive tissues, like that of non-orthodox seeds are only possible once the moisture content of explants is reduced to an optimal level with reasonably high viability and is able to survive freezing stresses (Pritchard *et al*. 2016). Various desiccation techniques for cryopreservation of desiccation-sensitive seeds, namely air

desiccation, pregrowth-desiccation, vitrification and encapsulation-dehydration are currently been used at cryolab of NBPGR (Chaudhury and Malik, 2014; 2016; Prakash *et al*. 2019 a, b). Vitrification and encapsulation has been attempted in *Artocarpus heterophyllus, Litchi chinensis*, *Poncirus trifoliata* and *Citrus* species (Malik and Chaudhury, 2006).

Using the cryoprotocols developed, the base collection of more than 20 indigenous species of 6800 accessions representing wide genetic diversity of fruits, nuts, agroforestry species, plantation crop species and industrial crops has been cryoconserved at National Cryogenebank (NBPGR Annual Report, 2018).

Determination of moisture content and air-desiccation

- Moisture content can be measured using low constant or high constant temperature oven method (ISTA, 2015). The weight of the sample container moisture bottles should be comparable to the explant to be weighed.
- In case of very small explants like embryos and embryonic axes, small vials made of aluminum foil can be used instead of glass weighing bottles.
- Whole seeds and embryos can be desiccated in the desiccator/ vacuum desiccator filled with charged silica gel. The duration of desiccation needs to be standardized depending upon the initial and desired moisture levels. After each desired desiccation level, explants may be packed in the cryovials and maintained in the desiccator till the germination and freezing steps are to be undertaken.
- In case of non-orthodox seeds, freezing can be attempted once they show the ability to tolerate desiccation down to 10-14% moisture content.
- The sensitivity to desiccation of seeds, embryos and embryonic axes can be evaluated by assessing the relation between the moisture content and the germinability of explants. The moisture content at which survival of seeds, embryos and embryonic axes is highest is the critical moisture content of that explant.
- For air-desiccation freezing excised embryonic axes may be kept in batches of 20-25 in the sterile air flow of laminar flow cabinet immediately after excision. Axes may be desiccated for 1 to 5h depending upon the size of axes, the initial and desired moisture content levels. After each desiccation interval, moisture content and viability of embryonic axes need to be determined. Viability of desiccated axes should be determined by culturing them in the prescribed culture media.
- If germinability of embryonic axes is lost on desiccation to moisture content range of 10 to 25%, then a higher or lower rate of drying or a different stage of maturity of explants may be explored. Alternative culture media may be tested for germination.

Storage in liquid nitrogen

- Freezing should be attempted at the critical moisture contents or between 10 to 25% moisture content, whichever is the lowest.
- A sufficient number of explants must be enclosed in cryovials or in sterile pieces of aluminum foil before immersion in liquid nitrogen. The freezing rate can be modified using various methods and containers. Thawing is to be performed in a water bath at the temperature of 37 to 40°C.

- Regrowth of explants is to be assessed by using *in vitro* culture methods for embryonic axes and embryos and ISTA methods for whole seeds. In case recovery growth is not upto the desirable level, the necessary changes in media and germination procedures must be made for optimum results.
- Production of morphologically abnormal seedlings should be noted as it may indicate stress of desiccation and / freezing or incorrect excision procedures.
- Occasionally, seeds of particular species may not respond well to NaOCl and/or to the commonly-used benomyl-based fungicides. In these cases other alternatives such as dilute solutions of 0.5-2% (w/v) calcium hypochlorite, 0.1-1% (w/v) mercuric chloride or 70% ethanol may be used.
- Embryo/axis are always limited in number when large experimentations are to be done. Hence fewer individuals per accession are usually cryostored. Additional explants will have to be added each year to the Cryobank.

2,3,5-Triphenyl tetrazolium chloride (TTC) test

In the TTC assay, also named as tetrazolium test, 2,3,5-Triphenyl tetrazolium chloride (TTC) is used to differentiate between metabolically active and inactive tissues. The white compound is enzymatically reduced to red TPF (1,3,5-triphenylformazan) in living tissues due to the activity of various dehydrogenases (enzymes important in oxidation of organic compounds and thus cellular metabolism), while it remains white in dead areas where these enzymes have been either denatured or degraded. Explants like seeds, embryos, embryonic axes are incubated in TTC solution 0.2- 0.6% (w/v) TTC in either water / phosphate buffer (0.05 M Na2HPO4–KH2PO4, pH 7.4). Dormant buds are incubated in this solution along with a 0.05% (v/v) wetting agent (Tween 20), and incubated in darkness at 30°C overnight. Thereafter, explants are rinsed using distilled water and observed under a stereoscopic microscope. TTC staining indicates viability.

Embryo cryopreservation (air desiccation-freezing)

Plant material

- *Citrus* spp.

Checklist of items required

- Sterile glass petridishes and 100 ml flasks
- Sterile filter paper discs
- Surgical instruments like scalpel blade holder, scalpel blades, forceps, needles, etc
- Sterile cryovials and cryomarkers
- MS culture medium in glass test tubes
- Dewar flasks containing LN2

Procedure

1. Collect seeds from ripe fruits.
2. Remove the seed coat from the seeds just before experimentation to obtain embryos.
3. Treat the seeds with bavistin solution for 2 minutes in lab.

4. Use for viability testing by petriplate germination/ *in vitro* culturing as per defined procedure of sterilization.
5. Desiccate embryos in the desiccator/ vacuum desiccator filled with charged silica gel. Standardize the duration of desiccation depending upon the initial and desired moisture levels. It could vary from 4 h to 48 h. Desiccate embryos to 10-14% moisture content.
6. After each desired desiccation level, pack embryos in the cryovials and maintain in the desiccator till the germination and freezing steps are to be undertaken.
7. Place about 10-15 desiccated embryos in 2/5 ml cryovials and plunge rapidly in liquid nitrogen (LN2).
8. Hold in LN for 48 h (for practical today do it for only 30 min) or more as per designed schedule.
9. Thaw the cryovials in a water bath at 38°C for 5 min.
10. Place seeds for petriplate germination.

Embryonic axes cryopreservation (air desiccation-freezing)

(after Malik and Chaudhury, 2010)

Plant material

- Apricot *(Prunus armeniaca)*

Checklist of items required

- Sterile glass petridishes and 100 ml flasks
- Sterile filter paper discs
- Surgical instruments like scalpel blade holder, scalpel blades, forceps, needles, etc
- Sterile cryovials and cryomarkers
- MS culture medium in glass test tubes
- Dewar flasks containing LN2

Procedure

1. Crack open the hard endocarp to extract the seeds.
2. Surface sterilize the seeds for 10-15 minutes in laminar flow cabinet as a standard procedure.
3. Rinse four times with sterile distilled water.
4. Remove brown seed coat and gently separate out the cotyledons snapping one of the attached connections with embryonic axes.
5. Make an incision at the other attached connection of embryonic axes to separate it out.
6. Use 15-20 axes for moisture content determination using low constant temperature oven method.
7. Spread rest of the axes on sterile filter paper discs in the air current of a laminar flow cabinet for desiccation to moisture levels between 6.8 to 7.5% (on fresh weight basis) as determined by low constant temperature oven method.

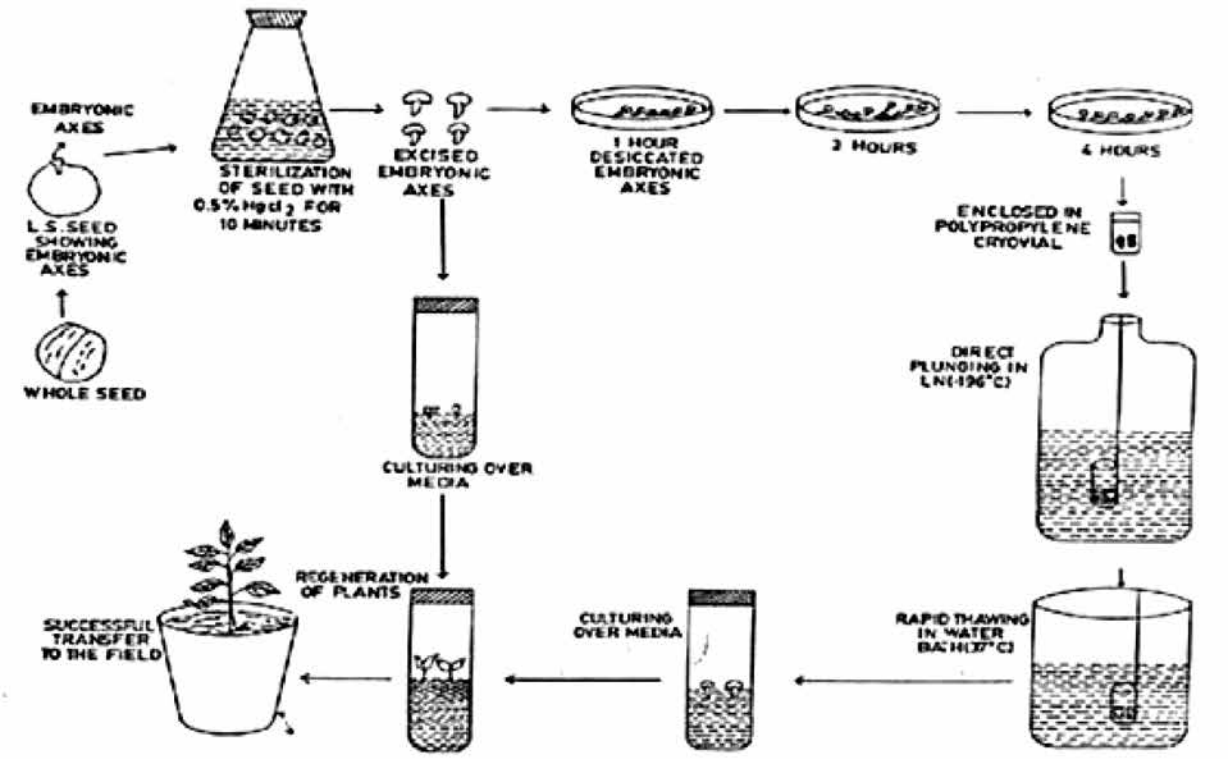


Fig. 1. *A generic protocol for air desiccation-freezing*

8. Place about 10-15 desiccated axes in sterile 2 ml cryovial and plunge rapidly in liquid nitrogen (LN).
9. Hold in LN for 48 hours (for practical today do it for only 30 min) or more as per designed schedule.
10. Thaw the cryovials in a water bath at +38°C for 5 min.
11. Culture the fresh axes in MS medium containing 0.1mg ml $^{-1}$ each of BAP and NAA and maintain at 25±2°C with a 16 h photoperiod under light intensity of 35 μE $m^{-2}s^{-1}$. Cryopreserved axes must be cultured in dark for one week before exposing to full light conditions.

Vitrification of embryonic axes

(after Malik and Chaudhury, 2006)

Plant material

- *Citrus* spp.

Checklist of items required

- Sterile glass petridishes and 100 ml flasks
- Sterile filter paper discs
- Surgical instruments like scalpel blade holder, scalpel blades, forceps, needles, etc
- Sterile cryovials and cryomarkers
- Preculture medium- basal MS medium supplemented with 0.3 M sucrose and 2M glycerol

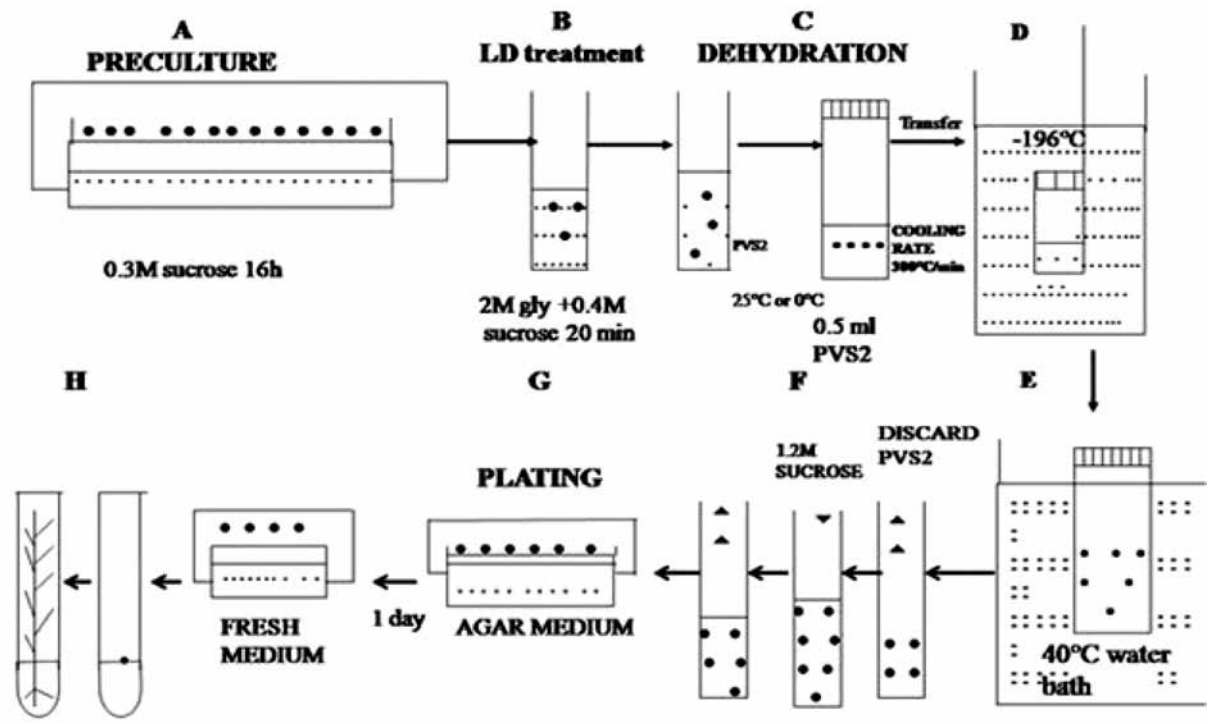


Fig. 2. *A generic protocol for vitrification of embryonic axes*

- Loading solution (0.4M sucrose, 2M glycerol in basal MS medium)
- PVS2 solution [30% (w/v) glycerol, 15% (w/v) ethylene glycol, 15% (w/v) Dimethyl sulphoxide]
- Unloading solution (1.2M sucrose in basal MS medium).
- Culture medium [MS medium macro- and micro-nutrients, vitamins, iron, supplemented with 1g l^{-1} activated charcoal, 0.17g l^{-1} NaH_2PO_4 and 1mg l^{-1} each of BAP and NAA after Chin *et al.*, (1988) *Cryoletters* 9: 372-379]
- Dewar flasks containing LN

Procedure

1. Surface sterilize the decoated seeds using sodium hypochloride for 10 minutes in laminar flow cabinet.
2. Rinse four times with sterile distilled water.
3. Gently separate out the cotyledons snapping one of the attached connections with the zygotic embryonic axes.
4. Scoop it out by making another incision at the other joining point.
5. Use 15-20 axes for moisture content determination using low constant temperature oven method.
6. Preculture the axes on basal MS medium supplemented with 0.3 M sucrose and 2M glycerol for 16 to 24h and maintain in culture room conditions.
7. Transfer 15-25 axes to 1.2 ml sterile cryovials and treat with 0.5 ml loading solution (0.4M sucrose, 2M glycerol in basal MS medium) for 20 min at 25°C.
8. Replace loading solution with 0.5 ml Plant Vitrification Solution 2 (PVS2) for 30 min at 25°C.

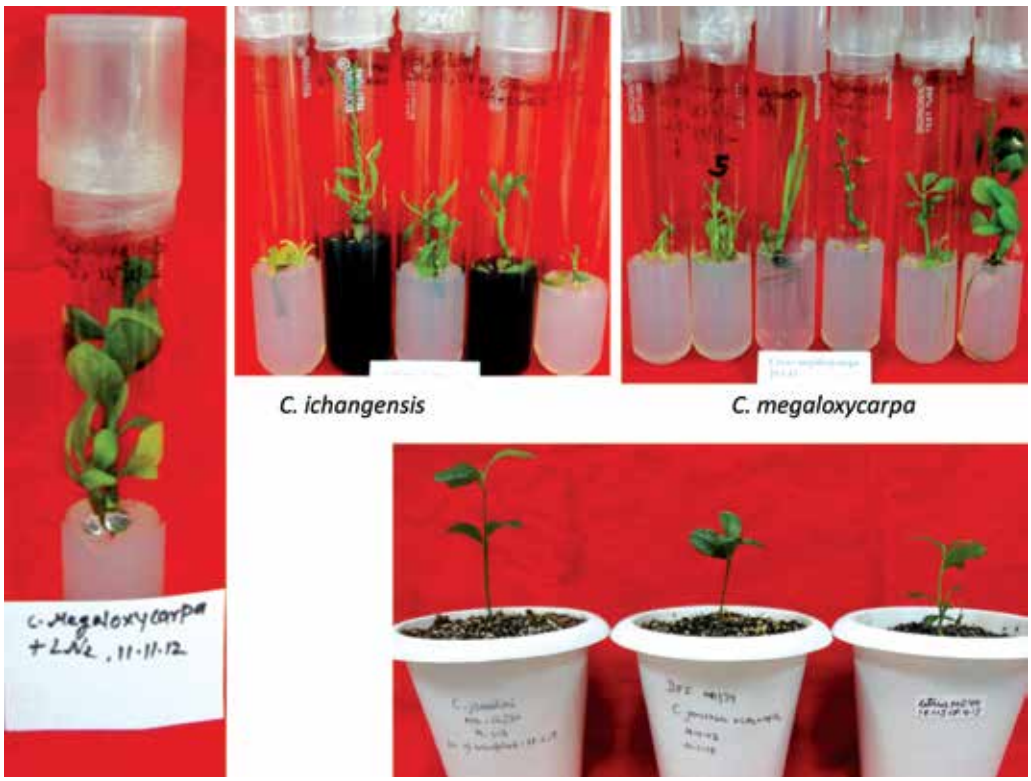


Fig. 3. *Successful recovery of plantlets from cryostored explants*

9. Thaw the cryovials in a water bath at 38°C±1°C for 1 min with vigorous shaking.
10. Immediately replace the PVS2 solution with 0.5 ml unloading solution (1.2M sucrose in MS basal medium). Leave the vials at 25°C for 20 min.
11. Drain the solution and blot the axes dry on sterile filter papers.
12. Culture the axes in MS medium macro- and micro-nutrients, vitamins, iron, supplemented with 1g l^{-1} activated charcoal, 0.17g l^{-1} NaH_2PO_4 and 1mg l^{-1} each of BAP and NAA and maintain at 25±2°C with a 16 h photoperiod under light intensity of 35 μE $m^{-2}s^{-1}$.
13. Plunge tightly closed cryovials containing axes in PVS2 solution rapidly in liquid nitrogen (LN2).
14. Hold in LN for 48h or more as per designed schedule.

Encapsulation-Dehydration of embryonic axes

Plant material

- *Citrus* spp.

Procedure

1. Aseptically excise the embryonic axes as described above.
2. Encapsulate in alginate beads by first suspending in calcium-free MS medium containing 3% (w/v) sodium alginate. Drops of this solution, each containing one EA, need to be dispensed with a pipette into MS basal liquid medium supplemented with 100mM calcium chloride.
3. Solidify the beads for 15 min at 25°C with occasional stirring.

4. Pre-culture the beads in liquid MS medium containing sucrose (0.30 to 1.0 M) on a rotary shaker for 20 h.
5. Remove beads from liquid medium and dehydrate at room temperature for 6 h in a laminar air flow cabinet.
6. Determine the moisture content of the beads.
7. Enclose the beads in 1.0 ml cryovials and plunge into liquid nitrogen.
8. Thaw the vials slowly at room temperature for 20 min and culture the beads *in vitro* as previously described.

Note

* *If the seeds are too hard and it is not easy to separate out their cotyledons, they may be first soaked in water for 20 minutes.*
* *The excision of attachment points of axis to cotyledons should be carefully done avoiding damages to shoot or root apex.*
* *Wear gloves while handling PVS2 in order to avoid any contact.*
* *The extraction of seeds from the ripe fruits may be completed within 4-5 days of harvest to obtain optimal results in cryopreservation.*

Selected references

Chaudhury R and Malik SK (2016) Expanding applications of cryobanking: meeting challenges for effective long-term storage. Ind. J. Pl. Gen. Res. 29(3): 303-306.

Chaudhury R and SK Malik (2014). Implementing cryotechniques for plant germplasm: storing seeds, embryonic axes, pollen and dormant buds. Acta Horticulturae. 1039: 273-280.

Chaudhury R, SK Malik, AP Singh, R Choudhary, D Pal and P Chaudhary (2011) Cryobanking of non-orthodox seed species. ICAR News. 17 (4): 4.

Chin HF, B Krishnapillay and ZC Alang (1988) Cryopreservation of Veitchia and Howea palm embryos: non-development of the haustorium. CryoLetters. 9: 372-379.

Hong TD and RH Ellis (1996) A protocol to determine seed storage behaviour. IPGRI Technical Bulletin No. 1. (J.M.M. Engels and J. Toll, vol. eds.) International Plant Genetic Resources Institute, Rome, Italy.

ISTA (2015) International Rules for Seed Testing. International Seed Testing Association, Switzerland.

Malik SK and R Chaudhury (2006) Successful cryopreservation of embryonic axes of two wild and endangered *Citrus* species of north eastern India for long term conservation. *Plant Genetic Resources: Characterization and Utilization*. 4 (3): 204-209.

Malik SK and R Chaudhury (2010) Cryopreservation of seeds and embryonic axes of wild apricot (*Prunus armeniaca* L.). *Seed Sci. Technol*. 38: 231-235.

Malik SK, R Chaudhury and HW Pritchard (2012) Long -term, large scale banking of Citrus species embryos: comparisons between cryopreservation and other seed banking temperatures. *CryoLetters*. 33: 453-464.

NBPGR Annual Report (2018) NBPGR, New Delhi.

Prakash K, R Chaudhury, MR Rohini, B Singh and B Malik, SK (2019b) Contrasting seed biology of two ornamental palms: Pygmy Date Palm (*Phoenix roebelenii* O'Brien) and Fishtail Palm (*Caryota urens* L.) and implications for long-term conservation. *Indian Journal of Traditional Knowledge*. 18(3):477-484.

Prakash, K, Sunil Kumar K. and Chaudhury, R. (2019a) Cryopreservation of kernel and zygotic embryos of oil palm (*Elaeis guineensis* Jacq). *Journal of Plantation Crops*. 47(1): 16-23.

Pritchard HW, J Nadarajan, D Ballesteros, K Thammasiri, S Prasongsom, SK Malik, R Chaudhury, HH Kim, L Line, WQ Lie, XY Yange and E Popova (2016) Cryobiotechnology of tropical seeds–scale, scope and hope. I International Symposium on Tropical and Subtropical Ornamentals, http://www.ishs.org/symposium/324. *Acta Horticulturae* 1167. ISHS2017. DOI 10.17660/ Acta Hortic. 2017.1167.6.

7. Cryopreservation of shoot apices by encapsulation – dehydration technique

Sandhya Gupta, Era Vaidya Malhotra, D.K. Nerwal, Suresh Mali and Shupra Deo

Introduction

Cryopreservation of shoot apices is useful for the long-term conservation of vegetatively propagated crops. Few techniques are available for cryopreservation of shoot tips, of which encapsulation-dehydration is a novel technique having several advantages. Although, both apical and axillary shoot tips can be used for this technique, actively growing apical shoot tips usually respond better. The technique of encapsulation-dehydration was utilized for the first time for cryopreservation of pear and potato shoot-tips (Dereuddre *et al.*, 1990; Fabre and Dereuddre, 1990). This method is based on encapsulation of explants in calcium alginate beads, followed by dehydration in high sucrose concentrations and desiccation to low moisture contents, to remove freezable water from plant tissues prior to liquid nitrogen exposure. This leads to vitrification of internal solutes, avoiding lethal intracellular ice crystallization. High survival rates and rapid growth of cryopreserved explants are observed using this technique. The technique has been applied to *in vitro* shoot tips of large number of temperate and tropical species.

Advantages

- No special equipment is needed, and
- Use of nontoxic cryoprotectant (mostly sucrose), which is non-hazardous to the human health.

Disadvantages

- Requirement of handling each bead several times, and
- Some plant species may show intolerance to the high sucrose concentrations.

Check list of items required

Plant material

Apical and axillary shoot tips from *in vitro* plantlets of *Humulus*, *Fragaria* and / or *Rubus*

Equipment and glassware

Binocular dissecting microscope, scalpel, forceps, Pasteur pipettes, cryovials, 90 mm and 60 mm Petri plates, Sieve, autoclaved flask and beaker, Petri dish lined with a filter paper.

Media/ Chemicals/ Reagents

Alginate, liquid culture medium containing 0.75 M Sucrose aliquoted (50 ml) into in 150 ml conical flasks, calcium-free liquid culture medium containing 3% (w/v) sodium alginate, liquid culture medium containing 100 mM $CaCl_2$ dispensed in 30 ml aliquots into 100 ml beakers, agar solidified recovery growth medium in petri plates or cell wells, Liquid nitrogen in a small bench top flask of 1 lit capacity.

Reagents

Sodium alginate solution (3%)

For preparing 100ml of alginate solution with 0.5 M sucrose:

1. Add required volume of MS stock solutions, except halides (Table 1), in a flask.
2. Dissolve 17.1 g of sucrose (0.5 M concentration) completely.
3. Make up the volume 50 ml using double distilled water and adjust the pH to 5.8.
4. Place the solution on a magnetic stirrer cum hot plate.
5. To dissolve 3 g of sodium alginate in the above solution, add it in small quantities with constant stirring till it dissolves completely.
6. Transfer the solution to a 250 ml bottle, autoclave and store for further use.

Calcium Chloride solution (100 mM)

For 500ml of $CaCl_2$ solution (100 mM):

(i) Dissolve 7.35 g of calcium chloride in liquid MS medium, without halides (Table 1).

(ii) Make up the volume to 500 ml using double distilled water and adjust pH to 5.8.

(iii) Dispense the solution in two bottles of 250ml each, autoclave and store for use.

Osmotic dehydration solution (0.75 M sucrose)

For 100ml of this solution:

1. Add the desired amount of all the MS stock solutions (Table 1) in a flask.
2. Dissolve 25.6g sucrose in the above solution.
3. Make up the volume to 100ml using double distilled water and adjust the pH to 5.8.
4. Autoclave the solution and store for future use.

Pretreatment medium (0.3M sucrose)

For 100 ml of this solution:

1. Add the desired amount of all the MS stock solutions (Table 1) in a flask.
2. Dissolve 10.27g sucrose in the above solution.
3. Make up the volume to 100ml using double distilled water and adjust the pH to 5.7.
4. Add 0.8g agar prior to autoclave.
5. Autoclave the solution and pour them onto 60 mm Petri Plate (20-25 ml/plate).

Encapsulation-dehydration procedure

Stock cultures

a. Humulus lupulus

One-month old *Humulus lupulus* cultures multiplied and maintained on MS medium supplemented with 0.1mg/l BAP, 3% sucrose and 0.5% clariagar.

b. Fragaria x ananassa

Three-week old *Fragaria* x *ananassa* cultures multiplied and maintained on MS medium supplemented with 1mg/l BAP, 1 mg/l IAA, 0.01 mg/l GA_3, 3% sucrose and 6 g/l agar.

c. Rubus

Three-week old cultures of *Rubus* sp. multiplied on MS mineral salts and vitamins with double EDTA-Fe, 2 mg/l BA, 0.1 mg/l IBA, 0.1 mg/l GA_3, 3.5 g/l agar, 1.45 g/l gelrite and 30 g/l sucrose at pH 5.7. Cold acclimatize *Rubus* plantlets for 3 weeks after the last subculture (22°C with 8 h light /-1°C 16 h dark) for 4 weeks.

Shoot tip excision

Excise shoot tips (about 2mm long), after removing the leaf primordia using a dissecting microscope from one-month old cultures. Incubate *Fragaria* shoot tips on a pretreatment medium (0.3M sucrose) overnight.

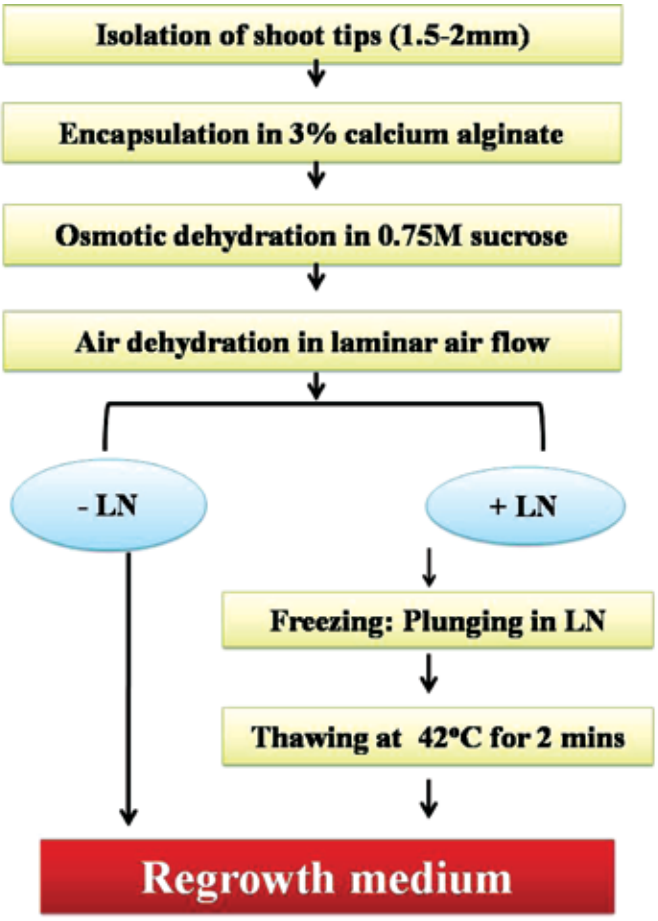


Fig. 1: *Generic protocol for cryopreservation of shoot tips by encapsulation – dehydration technique*

Encapsulation

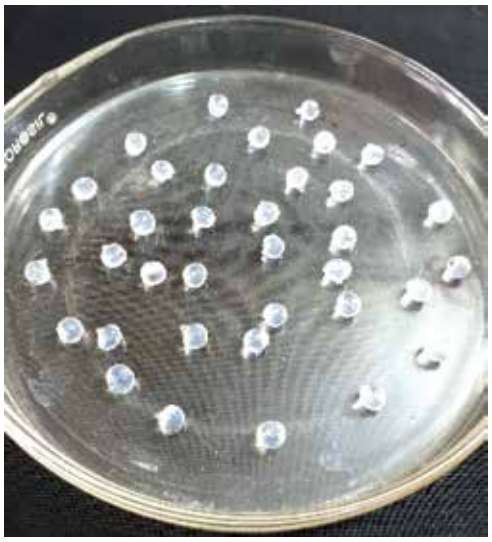
1. Suspend the excised shoot tips in 3% sodium alginate solution.
2. Using a sterile pasteur pipette, dispense individual shoot tips into liquid MS medium supplemented with 100mM calcium chloride.
3. The alginate polymerizes as calcium alginate forming beads enclosing the shoot tips.
4. Incubate for 20-30 minutes for complete polymerization.

Osmotic dehydration

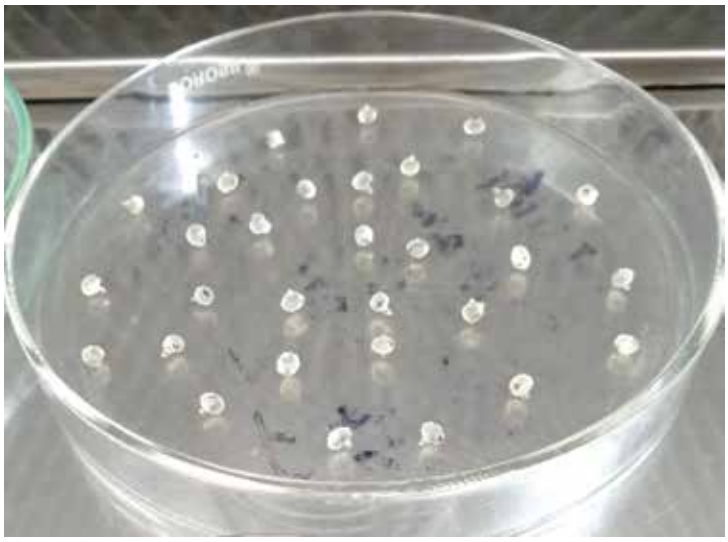
Suspend the beads in MS medium supplemented with 0.75M sucrose, for 24 hours for dehydration of the beads due to the higher osmoticum of the medium, thereby, increasing the concentration of the intracellular solutes due to sucrose uptake by the cells.

Air dehydration

Moisture content is further decreased by placing the osmotically dehydrated beads in sterile Petri plates in a laminar air flow for 4 hours.



Fresh beads



Beads after 3 h drying

Desiccation of beads can also be done on silica gel in small containers. However, it is essential to use a standardized system to use reproducible results.

Freezing

Transfer the beads to 1.8ml polypropylene cryovials and plunge into liquid nitrogen for rapid freezing.

Thawing and regrowth

1. Remove the cryovials from liquid nitrogen after 1 hour and thaw them by placing in a water bath maintained at 40°C for 2 mins.
2. Remove the beads from the cryovials and transfer them to the regeneration medium and incubate in dark for 3 days.

3. Remove the shoot tips from the beads and subculture on the same but fresh medium.
4. Observe the survival and regeneration of shoot tips. The shoot tips begin to show regrowth within 4 weeks of culture.
5. Survival and regrowth before and after LN treatment will be recorded.

Determination of moisture content

Moisture content of the beads is determined at three different stages.

Stage 1 – freshly encapsulated shoot tips

Stage 2 – shoot tips post osmotic dehydration

Stage 3 – shoot tips post air dehydration

For estimating the moisture content, measure the fresh weight of the beads using weighing bottles with 3 replicates with 5 beads each. To measure the dry weight, keep the bottles in a pre-heated oven at 103 ± 2°C for 17 hours for drying, and then determine the weight. Moisture content will be determined using the formula:

$$MC\ (\%) = \frac{\text{Friesh weight- Dry weight}}{\text{Fresh weight}} \times 100$$

Regeneration per cent

Viability of the shoot tips will also be determined at the same 3 stages as the ones used for determination of moisture content. At every stage the beads will be kept for regeneration on the regeneration medium and per cent regeneration estimated.

References

Dereuddre J, C Scottez , Y Arnaud and M Duron (1990) Resistance of alginate-coated axillary shoot tips of pear tree (*Pyrus communis* L. Beurrè Hardy) *in vitro* plantlets to dehydration and subsequent freezing in liquid nitrogen: effect of previous cold-hardening. *C. R. Acad. Sci. Paris* 310 Sér. III: 317-323.

Fabre J and J Dereuddre (1990) Encapsulation-dehydration: a new approach to cryopreservation of Solanum shoot-tips. *CryoLetters*. 11: 413-426.

Gupta S and B Reed (2006) Cryopreservation of shoot tips of blackberry and raspberry by encapsulation-dehydration and vitrification. *CryoLetters.* 27(1): 29-42.

8. Cryopreservation of wild and cultivated germplasm of *Musa*

Anuradha Agrawal, Shivani Singh, D.P.S. Meena, Hardeep Singh and Bart Panis

Introduction

Genus *Musa* L. of *Musaceae* includes both wild and cultivated forms of socio-economically important plants, belonging to nearly 70 species globally (Hakkinen 2013). It is considered as second largest fruit crop and ranked the fourth important food crop in terms of starch staple and cash crop in the world. Two species namely *M. acuminata* (contributing A genome) and *M. balbisiana* (contributing B genome) are believed to be of progenitors the banana crop. More than 1,000 cultivated varieties have been named so far, are mostly sterile and parthenocarpic triploids (2n = 3x = 33) with A & B genomes, as AAA (three copies of the haploid chromosome set) or AAB and ABB (mixtures of A and B). There are some 60 national collection centers for *Musa* globally, with a majority managing the collections in field gene banks (as live plants) and few as in vitro genebanks/ cryogenebanks. In India, nine field gene banks exist for *Musa*, the most important being at the National Research Center for Banana, Trichy, Tamil Nadu; Tamil Nadu Agriculture University, Coimbatore, Tamil Nadu and the Banana Research Stations at Kannara, Kerala and Jorhat, Assam. Together, the holdings at these field genebanks are more than 1,000 accessions (inclusive of duplicates).

A wide and promising approach to conserve *Musa* as vegetatively propagated species for long term, which offers an advantage of conserving germplasm without any alteration for unlimted period of time, is cryopreservation. Varied explants play an important role in cryopreservation, as in case of cultivated varieties, meristem used for cryopreservation but for conserving wild species, zygotic embryo have been used. The standardized protocols for cryopreservation in *Musa* (including bananas and plantains) have been used for varied explants such as embryogenic cell suspensions (Panis *et al*., 1990), proliferating meristem cultures (Agrawal *et al*., 2004, 2008; Panis *et al*., 1996, 2002), apical meristems excised from rooted *in vitro* plants (Thinh *et al*., 1999, Panis *et al*., 2005), seeds (Bhat *et al*., 1993; Chin 1996) and zygotic embryos (Abdelnour-Esquivel et al., 1992). Cryobanking seeds and zygotic embryos are options only for seed bearing wild species of *Musa*.

Presently, the In Vitro Gene bank (IVG) at ICAR-NBPGR, New Delhi, has more than 430 accessions of Musa spp. in the form of *in vitro* cultures, comprising both indigenous (65%) and exotic (35%) accessions. Out of these 85 accessions are cryobanked. Global collection of *Musa* is held at the *Musa* International Transit Centre (ITC), Bioversity International, hosted by the Katholieke Universiteit Leuven (KUL), Leuven, Belgium. It comprises some 1,479 accessions (including 220 wild taxa) in the form of in vitro cultures. So far,938 accessions are cryobanked, including 126 wild taxa.

In the following exercise, three types of explants (individual as well as proliferating meristems and zygotic embryos) would be cryopreserved using three methods – simple freezing, vitrification, droplet freezing and simple air dehydration.

Plant material

- Banana (*Musa sp.*) plantlets and mature seeded fruits of wild species of *Musa*.

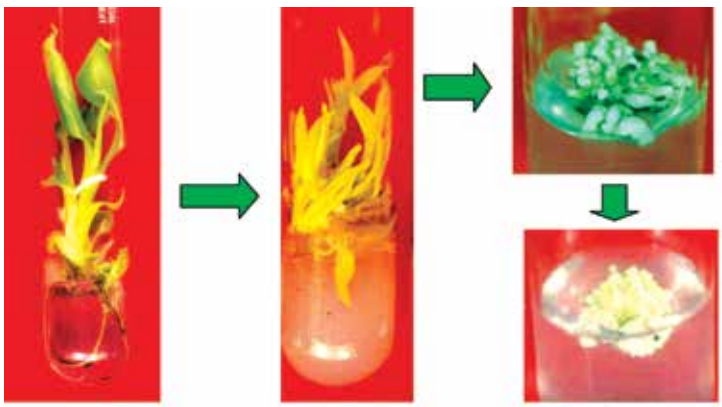
Equipment/ Glassware/ Plasticware

- Binocular dissection microscope
- Laminar air flow
- LN in Dewar flask
- Water bath at 40°C
- Cryovials (2 ml), Cryocanes
- Sterile Pasteur pipettes, Petri dishes (90 mm)
- Sterile containers for cryoprotectant solutions and liquid media
- Aluminum foil to wrap Petri dishes
- Sterile aluminum foil strips (30 × 5 mm)
- Sterile filter paper (cut to fit 60 mm and 90 mm Petri dishes)
- Cryovial box (12 × 12)
- Two Thermocol boxes with lids
- Tissue culture tools (forceps, scalpel, surgical blade No. 11 and 10), Timer, parafilm

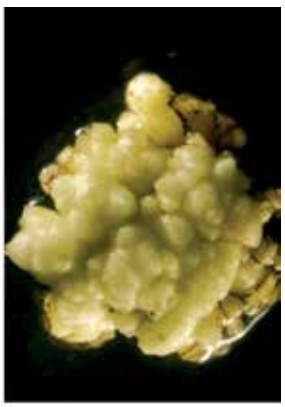
Method 1: Cryopreservation using proliferating meristems and simple freezing

1.1. Selection of plant material

- *In vitro* established cultures of banana growing on standard multiplication medium (Panis and Thinh, 2001) namely P5 medium, comprising MS salts (Murashige and Skoog, 1962)



Stages in development of 'cauliflower-like' meristems on P4 medium



A magnified view of 'cauliflower-like' meristems

+ 10 μM BA and 1 μM IAA + 10 mg/l ascorbic acid +3% sucrose + 0.25% gelrite, pH- 6.2 to be used.

1.2 Generation of proliferating meristems

- Starting with selected cultures, shoots to be sub-cultured on proliferation medium (P4 medium) with 100 μM BA at 4-6 weeks interval for 3-12 cycles, depending on the cultivar. White 'cauliflower-like' proliferating meristem clusters would be obtained.

1.3 Preculture of plant tissue

- Excise white meristematic clumps of about 4-6 mm diameter, each containing several apical domes. Culture these clumps on MS medium with 10 μM BA and 1 μM IAA (P5 proliferation medium) containing 0.4 M sucrose (136.92 g/l) for 2 weeks at normal growth conditions (25 ± 2°C and illumination of 50 $\mu Em^{-2}s^{-1}$).
- Growth and survival of meristem will determine visually, after 2 week pre culture period.

1.4 Dissection of explants

- Remove brown tissues and use only the healthiest meristems (white-yellowish) for further experimentation.
- Excise 30-100 meristems of 1 to 1.5 mm diameter (containing 3 to 6 meristematic domes), depending on cultivar and growth of meristematic clumps.

1.5 Direct freezing in liquid nitrogen

- Transfer about 8-10 excised meristematic clumps to 2 ml sterile cryovials.
- Close the vials and seal with Teflon tape.
- Pack the cryovials on aluminum cryocanes.
- Plunge the material into liquid nitrogen (LN) for storage for at least 1h.

1.6 Thawing

- Thaw the cryocanes with cryotubes rapidly by plunging in a water bath at 40°C for 1 min 20 sec.

1.7 Recovery and Regrowth

- Transfer the control (precultured but non-frozen) and frozen meristems (in Liquid Nitrogen) and semi-solid regeneration medium (MS + 3% sucrose + 1 μM BA).
- Incubation at stationery phase in dark for 7-8 days.
- After 7-8 days, cultures to be incubated under continuous light at 50 $\mu Em^{-2}s^{-1}$ at 25 ± 2°C.
- Observe and record growth after 4 weeks of freezing, using a binocular microscope.

Method 2: Cryopreservation using proliferating meristems and vitrification technique

2.1 to 2.4 Follow steps 1.1 to 1.4 as above.

2.5 Loading treatment

- Transfer all the excised proliferating meristems to 20 ml sterile plastic containers.
- Add about 5 ml loading solution (LS) to the excised meristems (60 - 80) for 20 min at room temperature. LS comprises 2 M glycerol + 0.4 M (13.68 g/100 ml) sucrose dissolved in MS medium (pH 5.8).

2.6 Dehydration with PVS2

- Remove the LS from the plastic container with a sterile Pasteur pipette.
- Add 5 ml of pre-chilled Plant Vitrification Solution (PVS2). PVS2 comprises 30% (3.26 M) glycerol + 15% (2.42 M) ethylene glycol + 15% (1.9 M) DMSO (Dimethylsulphoxide) in MS medium with 0.4 M sucrose (pH 5.8). As PVS2, is a high density chemical, proper mixing is required prior to use.
- Incubate the meristems in PVS2 by placing the plastic container on ice (≤ 0°C)* for a maximum of 2 h 30 min (duration is genotype-dependant). *Temperature should be maintained during incubation.
- Before the end of the incubation period, transfer the meristems from the PVS2 in the plastic containers into pre chilled cryotubes containing fresh PVS2.

2.7 Freezing

- Seal the cryotubes with Teflon tape (to prevent leaking during thawing).
- Transfer the cryotubes to aluminum cryocanes and plunge into LN.

2.8 Thawing and deloading

- For control, take out few meristems from the PVS2 solution (cryoprotected but non-frozen), and dipped into recovery solution (RS) for 15 min. RS comprises 1.2 M sucrose dissolved in MS medium (pH 5.8).
- Thaw the meristems by plunging the cryovials directly in a warm water bath (40°C) for about 1 min 20 sec with constant stirring of the cryocanes (all the ice needs to be melted)
- Clean the cryovials externally with ethanol and remove the Teflon tape.
- Remove PVS2 from the cryovials with Pasteur pipette.
- Add 1 ml of RS for 15 min at room temperature.

2.9 Recovery growth

- Transfer the meristems (control and frozen) from the RS to semi-solid recovery medium. The recovery medium comprises MS medium + 0.3 M sucrose + 1 μM IAA + 1 μM BA dispensed in Petri dishes (d=9 cm). Before culturing the explants, place two sterile filter paper discs on the medium (filter paper absorb excess RS from meristems).
- After 2 days transfer the meristems onto regeneration medium, without filter paper. The regeneration medium is identical to recovery medium except that sucrose is reduced to 3%.
- Incubate the cultures in dark for at least 1 week.
- Determine post-thaw recovery after 4-6 weeks.

Method 3: Cryopreservation using proliferating meristems and droplet freezing

3.1-3.6 Follow steps 2.1 to 2.6 as above

- Before completion of the incubation period, place 5-10 clumps of meristems in a droplet of the PVS2, kept on a sterile aluminum foil strip (25 × 5 mm). The foil should be held in a sterile Petri dish and placed on a pre-chilled (≤ 0°C) laptop cooler.

3.7 Fast-freezing

- Hold the aluminium foil containing meristems with a pair of forceps and plunge directly into LN. Transfer the foils in sterile cryovials (2 ml) held in a polycarbonate cryobox filled with LN.

3.8 Thawing and deloading

- Take out 1 or 2 aluminium foils with meristems and plunge into RS held in Petri dish and allow the treatment set for 20 min at room temperature.

3.8 Recovery growth

- Same as described under section 2.9

Method 4: Cryopreservation using individual meristems and droplet freezing

4.1: Selection of plant material

- The starting material comprises individual in vitro shoots of banana cultured on semi-solid MS medium supplemented with 1% activated charcoal for 4 weeks.

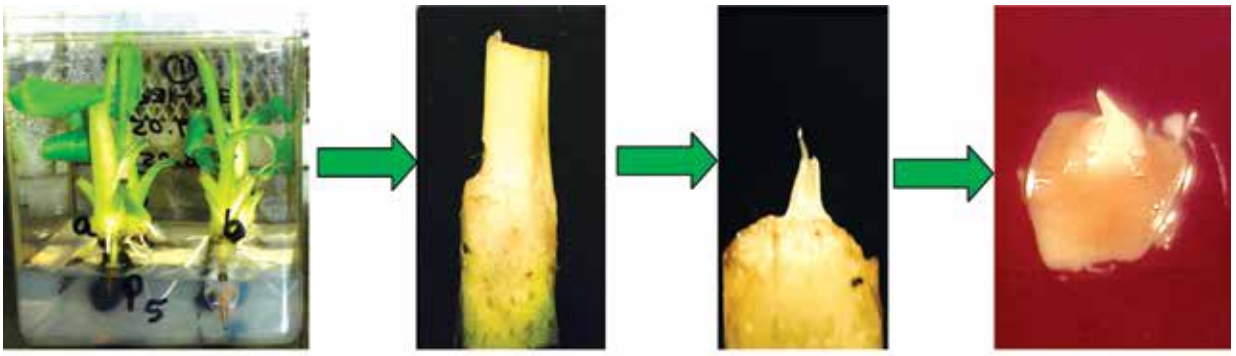
4.2 Preculture in sucrose

- The well-rooted shoots to be removed and dissected to obtain 4-6 cm long shoot. Culture the shoots in MS medium with 6% sucrose held in broad glass jars. About 4-6 plants per jar are to be raised.
- Cultures to be incubated for 4 weeks under light at 50 $\mu Em^{-2}s^{-1}$ at 25 ± 2°C.

4.3 Generation of individual meristems

- Isolation of individual shoot or explant using binolocular microscope.
- The explant comprises the meristematic dome along with one leaf primordia along with corm tissue (1 mm^3).
- The isolated meristems are to be placed on MS basal semi-solid medium, till the time of cryopreservation to avoid desiccation during isolation.
- After all the meristems are dissected, transfer to cryovials (10 meristems per vial).

Steps like loading treatment, PVS2 dehydration, freezing, thawing and recovery of growth are same as described under Method 3.



Rooted plantlets in sucrose (6%) medium

Stages in dissection of individual meristem

View of the meristem partially covered by leaf primordia, ideal for cryopreservation

Method 5: Cryopreservation using zygotic embryos and simple air dehydration-freezing.

5.1 Sterilization of seeds

- Manually extract seeds from mature fruits.
- Wash and clean seeds with Tween 20 (1-2 drops) in running water to remove pulp from the seeds.
- Sterilize the seeds with 1% $HgCl_2$ for 30 minutes.
- Dry the seeds for 14-16 hrs prior to remove excess moisture.
- Sterilization of seeds (acc. to sample size) can be repeated prior to further experimentation

5.2 Extraction of zygotic embryos

- Manually extract the zygotic embryos by cutting the seed diagonally, nearest to micropylar plug. Blade no. 10 use for seed cutting

5.3 Dehydration of zygotic embryos

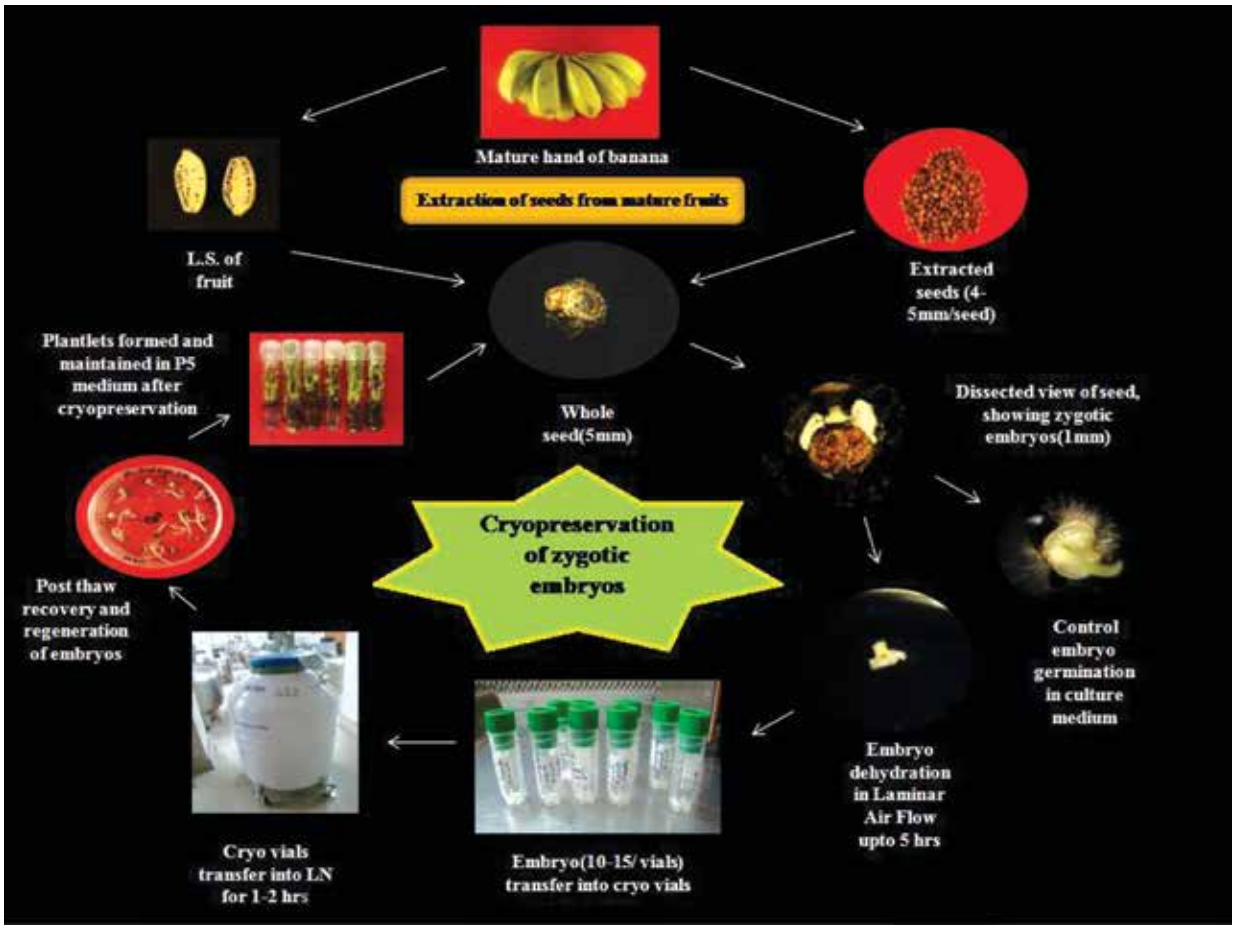
- Extracted zygotic embryos kept at sterile filter paper disc (d= 9cm) in light illumination of 50 $\mu Em^{-2}s^{-1}$ in laminar air flow for 4-5 hrs to get optimum dehydration.

5.4 Freezing

- Transfer 10-15 dehydrated embryos into 2 ml cryovial and seal them with cap.
- Directly plunge the cryovials into liquid nitrogen for 1-2 hours at -196°C.
- Maintain control non frozen dehydrated embryos at 25°C.

5.5 Thawing

- Take out the cryovials from Liquid Nitrogen box.
- Thaw them at 40°C for 1-2 minutes (prevent entry of water into the cryovials).



Zygotic Embryo Cryopreservation

5.6 Regeneration of zygotic embryos

- For regeneration, take out the zygotic embryos control as well as frozen.
- Inoculate them onto regeneration medium, consists of MS salts + 1μM BAP + 3% sucrose and 10 mg/l ascorbic acid, pH 6.2.
- Transfer the petri dshes in dark conditions initially for 15-20 days and afterwards in standard culture conditions at 25 ± 2% for maintenance.
- Record survival and regeneration after 15-20 days of inoculation.

Selected References

Abdelnour-Esquivel A, A Mora .and V Villalobos (1992) Cryopreservation of zygotic embryos of *Musa acuminata* (AA) and *M. balbisiana* (BB). *CryoLetters* 13: 159–164.

Agrawal A, R Swennen and B Panis (2004) A comparison of four methods for cryopreservation of meristems in banana (*Musa* spp.) *CryoLetters* 25: 101-110.

Agrawal A, RK Tyagi and R Goswami (2008) Cryopreservation of subgroup Monthan (ABB) of Indian cooking bananas (*Musa* spp.) *Curr. Sci.* 94: 1125-1128.

Agrawal A, RK Tyagi, , R Goswami, S Uma, MS Saraswathi and P Durai (2011) Cryobanking of banana (Musa sp.) germplasm in India: Evaluation of agronomic and molecular traits of cryopreserved plants. *Acta Horticulturae* 908: 129-138.

Agrawal A, R Sanayaima, R Singh R Tandon, S Verma and RK Tyagi (2014) Phenotypic and molecular studies for genetic stability assessment of cryopreserved banana meristems derived from field and in vitro explants sources. *In Vitro Cellular and Developmental Biology – Plant* 50: 345-356.

Bhat SR, KV Bhat and KPS Chandel (1997) Studies on germination and cryopreservation of *Musa balbisiana* seed. *Seed Sci. Technol.* 22: 637-640.

Chin HF (1996) Germination and storage of banana seeds. In: Frison EA, Horry JP, Waele D De (eds), Proc. *New Frontiers in Resistance Breeding for Nematode, Fusarium and Sigatoka*, Kuala Lumpur, Malaysia, pp. 218–227.

Häkkinen M (2013). Reappraisal of sectional taxonomy in *Musa* (Musaceae). *Taxon*, 62(4), 809-813.

Panis B (2009) *Cryopreservation of Musa Germplasm*, 2nd edition. Technical Guidelines No. 9 F. Engelmann and E. Benson (Eds.), Bioversity International, Montpellier, France, 48 p.

Panis B and NT Thinh (2001) *Cryopreservation of Musa Germplasm. INIBAP Technical Guidelines 5*. Escalant J.V. and Sharrock S. (eds.), International Network for the Improvement of Banana and Plantain, Montpellier, France, 44 p.

Panis B, B Piette and R Swennen (2005) Droplet vitrification of apical meristem: a cryopreservation protocol applicable to all Musaceae. *Plant Sci.* 168: 45-55.

Panis B, H,Strosse I Van Den Hende and R Swennen (2002) Sucrose preculture to simplify cryopreservation of banana meristem cultures. *CryoLetters* 23: 375-384.

Panis B, N Totté, K Van Nimmen, LA Withers and R Swennen (1996) Cryopreservation of banana *(Musa* spp.) meristem cultures after preculture on sucrose. *Plant Sci.* 121: 95-106.

Panis B; LA Withers and EAL De Langhe (1990) Cryopreservation of *Musa* suspension cultures and subsequent regeneration of plants. *CryoLetters* 11: 337-349.

Thinh NT, H Takagi and S Yashima (1999) Cryopreservation of *in vitro*-grown shoot tips of banana (*Musa* spp.) by vitrification method. *CryoLetters* 20: 163-174.

9. Cryopreservation of Pollen

Gowthami R., Vartika Srivastava, Anang Pal Singh and Hardeep Singh

Introduction

Pollen from most plant species are short lived after dehiscence and hence, it is very important to conserve these to overcome seasonal, geographical and physiological limitations for hybridization with suitable female parents. Pollen conservation is complementary to seed or vegetative propagule conservation and hence pollen banks are generally established as supplements to the genebanks. In the present scenario, cryopreservation is the only option available for long-term conservation and is widely used for conservation of food crops, fruits, vegetables, ornamentals, etc. in the form of seeds, embryonic axes, embryos, *in vitro* grown explants, pollen and genomic resources. A pollen cryobank can provide a constant supply of viable and fertile pollen to allow supplementary pollinations for breeding programs and can become an important component of national gene banks. Pollen cryopreservation methods have been reported for more than 170 species, including fruits, forest trees, staple crops, vegetables, forage grasses, ornamentals, economic and medicinal plants.

At the Cryogenebank of ICAR–National Bureau of Plant Genetic Resources (NBPGR), New Delhi, India, so far, a total of 576 accessions in the form of pollen grains belonging to different crops, such as, bitter gourd (*Momordica charantia*), cherry (*Prunus nepalensis*), citrus (*Citrus indica*), coconut (*Cocos nucifera*), cowpea (*Vigna unguiculata*), cucumber (*Cucumis sativus x hardwickii*), Indian mustard (*Brassica juncea*), kokum (*Garcinia indica*), litchi (*Litchi chinensis*), long melon (*Cucumis melo var. utilissimus*), maize (*Zea mays*), malabar tamarind (*Garcinia gummi gutta*), mango (*Mangifera indica*), mangosteen (*Garcinia mangostana*), mustard *Brassica sp.*), okra (*Abelmoschus spp*), onion (*Allium* spp.), oil palm (*Elaeis guineensis*), pumpkin (*Cucurbita moschata*), radish (*Raphanus sativus*), rapeseed (*Brassica rapa var. brown sarson*), snapmelon (*Cucumis melo var. momordica*), sweet gourd (*Momordica cochinchinensis*), sesame (*Sesamum indicum*), toria (*Brassica rapa var. toria*), watermelon (*Citrullus vulgaris*), wheat (*Triticum durum*), wild vigna (*Vigna trinervia var. bourneae*), yellow mustard (*Brassica campestris var. yellow sarson*), wild barley (*Hordeum spontaneum*) etc. are conserved.

Aim of pollen cryopreservation

The main objective of pollen cryopreservation is to serve plant breeders who attempt hybridization between plants cultivated/grown in different geographic regions, or showing non-synchronous flowering.

Significance of pollen cryopreservation

- Ensures the availability of male gametes for hybridization program without the need to grow male parent separately
- Availability of pollen irrespective of season and weather helps in overcoming problems of synchronous flowering
- Facilitates the supplementary pollinations in orchard crops
- A continuous source of pollen for studying pollen biology
- Pollen storage is required for those crops that have recalcitrant seeds and exhibit poor response to tissue culture methods

Generic procedure for pollen cryopreservation

The various steps involved in pollen cryopreservation procedure are: collection and extraction of pollen, assessment of pollen viability, cryostorage and post-viability/fertility assessment of cryostored pollen.

Pollen collection and extraction

Collection and extraction of pollen grains from inflorescence in a viable condition is the basic requirement of conservation and utilization of pollen. The procedures involved in pollen collection depend on the type of species, inflorescence, and peak anthesis period. Pollen viability varies from species to species, among different accessions in a species, among individuals in an accession, pollen collected during different periods of flowering, at different times of the day etc. It may be due to environmental changes, genotypic differences and physiologic status of plants. In general, shelf life of pollen collected from immature, aged, or weather-damaged anthers is short. Thus, pollen should be harvested during peak flowering period and soon after anthesis, usually in the morning hours (8 – 10 A.M.) on a bright sunny day. Pollen can be collected from dehiscing anthers by gently tapping with a needle over a butter paper sheet. When anthers are not properly dehisced they are left under a table lamp for dehiscing. In case of sticky pollen, they can be extracted using organic solvents such as cyclohexane or hexane for 2-3 min (to remove the sticky pollen coat substances).

Pollen viability assessment

Monitoring pollen viability is a pre-requisite factor, before pollen is either used in breeding or conservation. From time to time many pollen viability tests have been standardized, like staining techniques to differentiate sterile and fertile pollen (TTC test, Fluorochromatic Reaction (FCR) test), *in vitro* germination techniques (hanging drop technique, sitting drop method, modified cellophane technique, suspension culture, surface culture and fertilizing ability (fruit and seed set).

A. Staining

a) Tetrazolium (TTC) test

It is the most commonly used staining test to check pollen viability. Tetrazolium test is based on the reduction of soluble colourless tetrazolium salt to reddish insoluble formazan in the presence of dehydrogenase. Viable pollen grains take a reddish colour when incubated

in tetrazolium solution (0.1-1%) for 30-60 minutes at 30-37°C due to the formation of formazan.

b) Fluorochromatic Reaction (FCR) test or Fluorescein Diacetate Test (FDA)

In this test, pollen grains are incubated in fluorescein diacetate (FDA) solution, where the non-polar non-fluorescent FDA enters the pollen cytoplasm. Esterases of cytoplasm hydrolyze FDA and release fluorescein, which is polar and fluorescent. Fluorescein accumulates in the cytoplasm of viable grains and gives green or yellowish-green fluorescence within 1-2 min.

B. *In vitro* germination

In vitro pollen germination is the most commonly used viability testing method in pollen physiology. This technique provides a simple experimental method to study the physiology and biochemistry of pollen germination and pollen tube growth. Within a few hours, pollen tubes grow *in vitro*. The composition of a germination medium to obtain optimal response has to be empirically formulated for each species. The liquid nutrient medium needs to be prepared in deionized, double distilled water with pH 7.3. Compositions of 3 different commonly used pollen culture media are given in Table 1.

Table 1. Composition of pollen culture media

Ingredients	Brewbaker & Kwack's Medium	Roberts' Medium	Hodgkin & Lyon's Medium
Sucrose	10%	20%	20%
Boric acid	100 mg/1	10 mg/1	100 mg/1
Calcium nitrate	300 mg/1	-	400 mg/1
Calcium chloride	-	362 mg/1	-
Magnesium sulfate	200 mg/1	-	200 mg/1
Potassium nitrate	100 mg/1	100 mg/1	100 mg/1
Tris	-	60-130 mg/1	-
TAPS	-	-	4.86 g/l

a) Hanging Drop Culture (Cavity slide method)

The hanging drop culture method is used with liquid pollen germination medium. A drop of pollen germination medium is placed in a cavity slide and pollen is dusted over and covered with coverslip with its periphery sealed with vaseline.

b) Sitting Drop Culture

The sitting drop culture method is one of the most commonly used methods and is simpler than the hanging drop method. In this method a drop of the liquid culture medium is placed on a slide and pollen is dusted on the drop. The culture is then maintained in a humid chamber to prevent evaporation.

c) Suspension Culture

This method is suitable for large samples. Pollen grains are dusted in a vial containing 2 to 10 ml culture medium and incubated on a shaker and observed for germination by placing a drop on a slide.

d) Surface culture

Pollen grains of many species germinate better on the surface of agar/agarose/gelatin medium or on cellophane placed in contact with culture medium than in liquid medium.

C. *In vivo* pollen germination and pollen tube growth

Vigor of sample pollen can also be determined by pollinating them on pistils along with fresh pollen and measuring the pollen tube growth at regular intervals.

D. Fertilizing ability

It is the best method to test the viability by controlled pollinations in the field based on seed set and fruit set percentage.

Cryopreservation

Pollen with high moisture levels does not survive immersion to low temperatures due to intracellular ice formation. Generally, pollen which is bicellular at anthesis is usually shed at lower moisture content, survives extensive desiccation, is longer – living and can be stored without desiccation or partial desiccation. Tricellular pollen at anthesis has high moisture content and survives only limited desiccation. Desiccation-tolerant pollen with low moisture levels usually has viability after cryopreservation. In a practical sense, desiccation-sensitive pollen can be cryopreserved by partially desiccating pollen to moisture levels where no freezable water exists but above levels where desiccation injury is apparent.

Determination of moisture content and desiccation

Moisture content can be measured using a low constant or high constant temperature oven method (ISTA, 1999). The weight of the empty moisture bottles or aluminum foil vials should be comparable to the weight of explants to be weighed. Desiccation of pollen with higher moisture content is usually carried out on charged silica contained in airtight desiccators for 2-3 hours. Rapid air-drying can also be achieved by using specialized pollen-dryers that blow air at 20% to 40% RH and 20°C, to quickly reduce moisture content. Regular monitoring of the sample should be done to ensure high viability and low moisture content. Excessive desiccation can lead to a loss in viability.

Storage in LN

Pollen desiccated to suitable moisture contents can be stored in aluminum packets, gelatin capsules or cryovials. Pollen in sufficient quantities can be easily packed in cryovials of 1 to 2 ml capacity and stored at –196°C (under liquid nitrogen) by placing into a canister. Enough replicates (10) of each pollen sample need to be cryostored.

Thawing

Cryovials may be removed from liquid nitrogen and kept at room temperature for 30 min or can be thawed in water bath at 30 – 40°C.

Cryopreservation of *Allium* spp. pollen

Checklist of items required

Petri dishes, butter paper, forceps, clean razor blade, needle, smooth paintbrush, cryovials, aluminum strips, glass slide, needle, microscope

Pollen germination medium:

Chemical	Concentration
Sucrose	10%
Boric acid	100 mg/1
Calcium nitrate	300 mg/1
Magnesium sulfate	200 mg/1
Potassium nitrate	100 mg/1

Procedure

1. Collect umbels (inflorescence in *Alliums*) with partial bloom flowers from the field on a sunny day between 9 to 10 am.
2. Tap the umbels gently using a needle on clean petri dishes with butter paper discs. If anthers are not properly dehisced, leave them under a table lamp for dehiscing.
3. Remove floral debris and consolidate the pollen using a brush.
4. Transfer the collected pollen to aluminum pouches.
5. For checking the viability of the non-frozen fresh pollen before plunging into liquid nitrogen, place two drops of pollen germination medium on a clean glass slide using a dropper or Pasteur pipette.

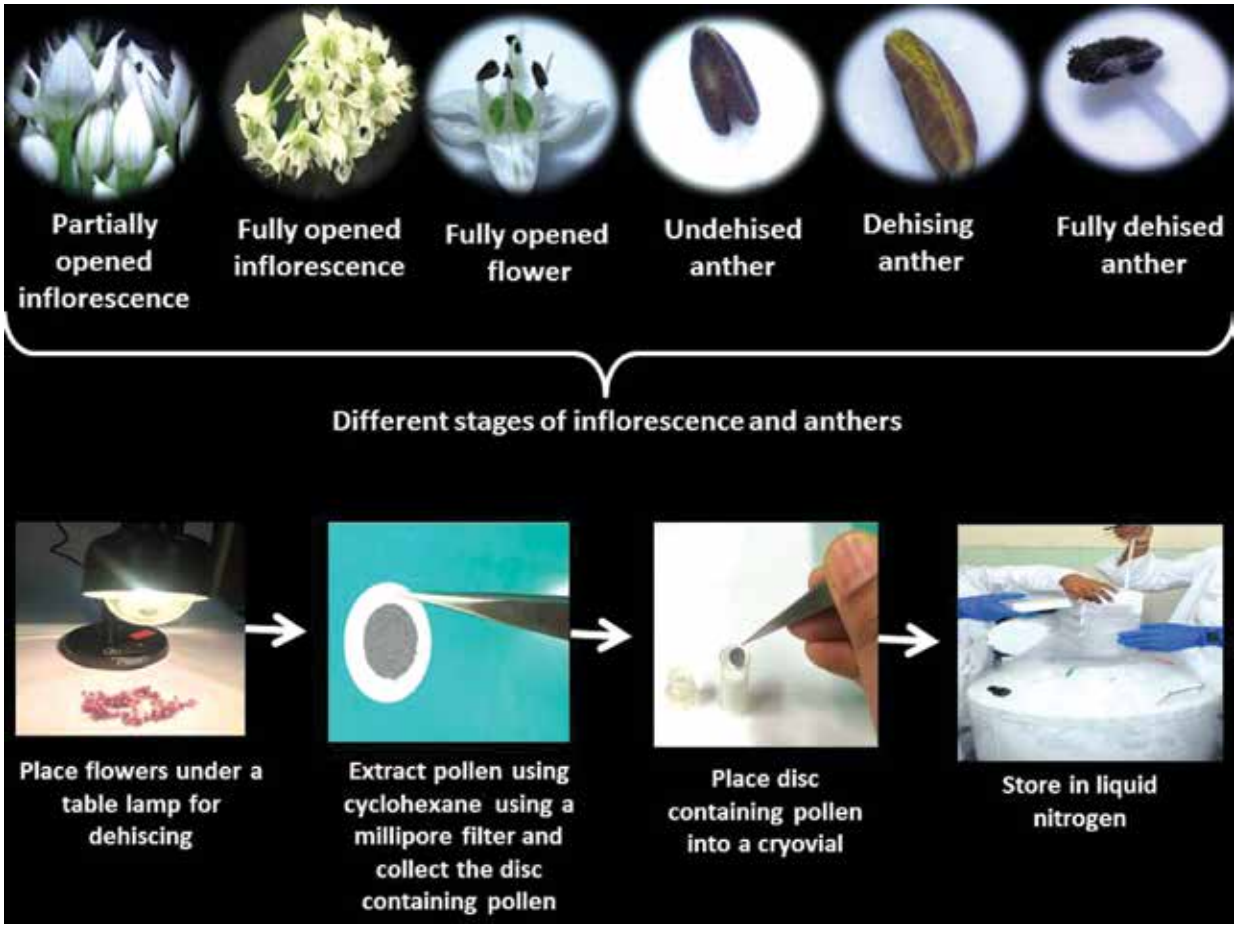


Fig. 1. *A generic procedure for Allium spp. pollen cryopreservation*

6. Take out pollen from the aluminum pouches and dust a few pollen grains on the drop on the slide.
7. Smear the pollen grains uniformly.
8. After 1 hour, place the glass slide on the stage of the compound microscope and count the number of pollen grains. A minimum of 400-500 pollen grains can be counted in 10 randomly selected microscopic fields. A pollen grain is considered germinated when pollen tube length (PTL) is at least equal to or greater than the grain diameter.
9. Percent pollen germination is calculated by formula:

$$\text{Pollen germination (\%)} = \frac{\text{No. of germinated pollen per field of view}}{\text{Total number of pollen grains per field of view}} \times 100$$

10 Desiccate the pollen in silica gel (based on moisture content)

11. For cryopreservation, label the cryovials and place aluminum pouches with pollen into the cryovials.
12. Plunge the cryovials with pollen in liquid nitrogen.
13. For thawing and viability assessment, remove the cryovials from the liquid nitrogen and leave it in ambient temperature for 10- 20 min. Record the post LN viability.

Cryopreservation of *Abelmoschus* spp. pollen

Checklist of items required

Petri dishes, butter paper, forceps, clean razor blade, needle, smooth paintbrush, cryovials, aluminum strips, glass slide, needle, syringe, filter unit, filter disc (pore size 0.22μm), microscope.

Pollen germination medium:

Chemical	Concentration
Sucrose	15 - 20%
Boric acid	100 mg/1
Calcium nitrate	300 mg/1
Magnesium sulfate	200 mg/1
Potassium nitrate	100 mg/1

Procedure

Cryopreservation

1. Collect opening flowers from the field at 9 to 10 am.
2. Remove petals and separate the anthers.
3. Place anthers in to a glass vial (10 ml capacity) using forceps.
4. Add 5 ml of cyclohexane (organic solvent) and shake vigorously for 50 sec to 1 min.
5. Draw the pollen suspension directly (*i.e.*, without using a needle) into a disposable syringe.

6. Attach the pollen suspension filled syringe to the assembled filter unit with filter paper discs.
7. Press the syringe and pass the suspension through the filter.
8. Detach the syringe from the filter unit and dismantle the filter unit.
9. Remove the empty anthers and anther debris retained on the filter disc.
10. Collect the disc containing pollen and place it in a cryovial or separate the pollen from the disc using a smooth paintbrush and transfer the pollen to aluminum pouches.
11. Check the viability of the non-frozen fresh pollen before plunging into liquid nitrogen as described in the procedure for pollen of *Allium* species.
12. Place aluminum pouches with pollen into labelled cryovials.
13. Plunge cryovials with pollen in liquid nitrogen.
14. For thawing and viability assessment, remove the cryovials from the liquid nitrogen and leave it in ambient temperature for 10- 20 min. Record the post LN viability.

Post-storage fertility assessment

a) Emasculate the flowers in the field
b) Pollinate the emasculated flowers with LN stored pollen.
c) Observe the normal seed and fruit set.

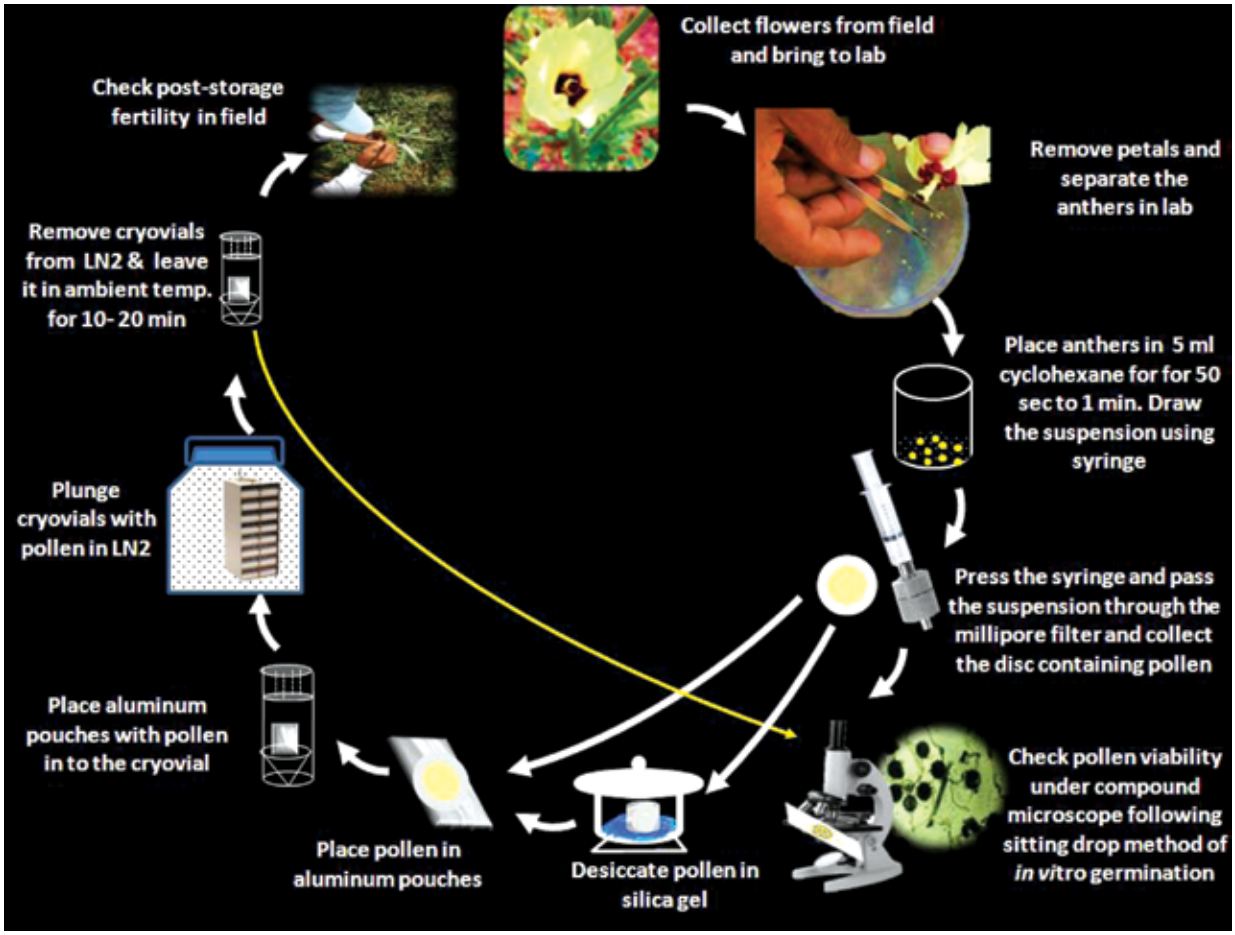


Fig. 2. *A generic protocol for Abelmoschus spp. pollen cryopreservation*

Note

* *Pollen should not be collected from infected or insect pest-damaged flowers*
* *Pollen should not be collected on a rainy day or if it had rained overnight*
* *Pollen should not be forced from anthers*
* *Pollen should be free of anther debris*
* *Sufficient amounts of pollen must be gathered and processed for banking purposes*

Selected references

Chaudhury R and SK Malik (2017) Cryopreservation of plant species: practical approaches from handling to cryobanking. Indian Council of Agricultural Research (ICAR)-National Bureau of Plant Genetic Resources, Pusa Campus, New Delhi - 110012. 52 p.

Chaudhury R, SK Malik, J Joshi and Shivanna KR (2010) Cryoconservation of pollen for germplasm storage and for utilization in breeding programmes. *J. Palynology*. **46**: 205-218.

Chaudhury R, SK Malik and S Rajan (2010) An improved pollen collection and cryo preservation method for highly recalcitrant tropical fruit species of mango (*Mangifera indica* L.) and litchi (*Litchi chinensis* Sonn.). *Cryo Lett*. **31(3)**: 268–278.

ISTA (1999) International rules for seed testing. Rules 1999. *Seed Sci Technol.* **27**: 1–133 (Supplement).

Shivanna KR and NS Rangaswamy (1992) Pollen Biology: A Laboratory Manual, Springer-Verlag, Berlin, 119 pp.

10. Cryopreservation techniques in Alliums

Ruchira Pandey, R Gowthami, Neelam Sharma, Ramesh Chandra and Anuradha Agrawal

Introduction

A viable and promising option, cryopreservation holds the key for long-term conservation of vegetatively propagated plant species. Having been advocated as the ultimate preservation method, the non-lethal storage of living tissues at ultra low temperatures (usually below -130°C), most commonly at the temperature of liquid nitrogen (LN2;-196°C) offers the advantage of preserving material for unlimited period without alteration. Success of any cryopreservation protocol depends mainly on avoidance of ice crystal formation within cells of biological tissues during rapid cooling in liquid nitrogen. Consequently, free water in cells needs to be reduced prior to freezing, to avoid crystallization and prepare these cells to vitrify upon LN freezing.

Most hydrated tissues cannot tolerate dehydration to the desired moisture level (ca. 20-30%), to avoid crystallization during freezing. The key to successful cryopreservation, is dependent mainly, on induction of dehydration tolerance. The new, vitrification-based, cryopreservation protocols, rely mainly on elimination of most or all freezable water from explant tissues by means of physical or chemical dehydration. To achieve this, dehydration of explants (which need to be preserved) is carried out employing highly concentrated vitrification solution. Additionally, pretreatment procedures can enhance dehydration tolerance leading to viable freezing during liquid nitrogen exposure. Chemical cryoprotection employs a chemical or a mixture of chemicals (cryoprotectant) which protect tissues under conditions of exposure to ultra low temperatures.

A variety of compounds (sucrose, glucose, proline, mannitol, glycerol, sorbitol, trehalose, polyethylene glycol, ethylene glycol) are used to cryoprotect plant tissues. Vitrification solutions contain two or more of the above-mentioned components which are applied either at 0^0C or at 25^0C and at greater concentrations (15-30% (v/v) or (w/v). The additives are generally toxic at these concentrations hence duration of exposure is very crucial.

Amongst bulbous crops particularly in alliums, *Allium sativum* (garlic) has been used as a model system to develop a workable cryopreservation protocol using vitrification (V) or droplet vitrification (DV) technique. The latter technique has proved to be more successful with respect to post-thaw regeneration. Interestingly, cryopreservation of garlic has been reported with two types of cryoprotectant solutions with percent regrowth varying with the genotype. In the following exercise, V and DV protocol for garlic shoot tip cryopreservation has been outlined.

Plant material

- Garlic (*Allium sativum*) cloves

Equipment/ Glassware/ Plasticware

- Binocular dissection microscope with light, B.O.D. Incubator, Laminar air flow, LN in Dewar flask, Water bath at 37°C
- Cryovials (2 ml), Cryocanes, Cryo vial holder (12 ° 12)
- Sterile Pasteur pipettes, Petri dishes (35mm, 60mm and 90mm)
- Sterile containers for cryoprotectant solutions and liquid media
- Sterile aluminum foil strips (30 × 5 mm), filter paper (cut to fit 60 mm and 90 mm Petri dishes)
- Two Thermocol boxes with lids
- Tissue culture tools (small soft forceps, scalpel and surgical blade No.11), Parafilm, Cork borer of 3 mm diameter, Aluminum foil to wrap Petri dishes

Chemicals/ Reagents

- Pre culture medium (MS basal medium containing 0.3M sucrose)
- Cryoprotectant solutions: (i) PVS3 solution comprising 50% sucrose (w/v) and 50% glycerol (w/v) in MS liquid medium; (ii) PVS2 solution (Sakai *et al.*, 1990) containing 30% (w/v) glycerol, 15% (w/v) ethylene glycol, 15% (w/v) DMSO and 0.4 M sucrose in MS liquid medium at pH5.8.
- Unloading solution (MS liquid medium with 1.2 M sucrose) at pH5.8
- Recovery medium (B5 + 0.1 mg/l NAA + 0.5 mg/l 2iP)

Vitrification protocol

a) Preparation and surface sterilization of explants

1. Isolate cloves of garlic from dormant bulbs and remove outer dry protective sheath.
2. Extract meristem tips from garlic cloves using a borer with a diameter of 3 mm.
3. Give a transverse cut to the tips at 3-4 mm height, on a sterile Petri dish and trim the basal plate to 0.5 to 1.0 mm thickness with a sterile surgical blade No.11. The explants employed for freezing comprise the meristematic dome, the surrounding leaf primordia and a basal part.
4. For surface sterilization, treat the meristem tips with rectified for 1 min and then disinfect with 0.1% $HgCl_2$ for 3 min.
5. Rinse with sterile distilled water 3-4 times under aseptic conditions.

b) Preculture of excised shoot tips

1. Implant sterilized shoot tips (measuring 3-4 mm), on pre culture medium (containing 0.3 M sucrose in MS basal medium) in disposable, sterilized Petri dishes (60 mm diameter).

2. Cold-harden the explants at 10°C under a 16 h light/8 h dark photoperiod for 2-3 days in an incubator.

c) Dehydration with PVS2/PVS3

1. Following pre culture, transfer 10 explants each to 2 ml cryovial containing 1 ml PVS2 /PVS3 solution.
2. Maintain these vials at room temperature for 40 min for PVS2 and 120 min for PVS3. Prior to freezing, replace old PVS2 /PVS3 respectively, with fresh solution (1ml).

d) Freezing in liquid nitrogen

1. Following cryoprotective dehydration with PVS2/PVS3, plunge one cryovial at a time, into liquid nitrogen using a forceps and hold for 1 h at -196°C.

   Maintain non-frozen controls at 25°C.

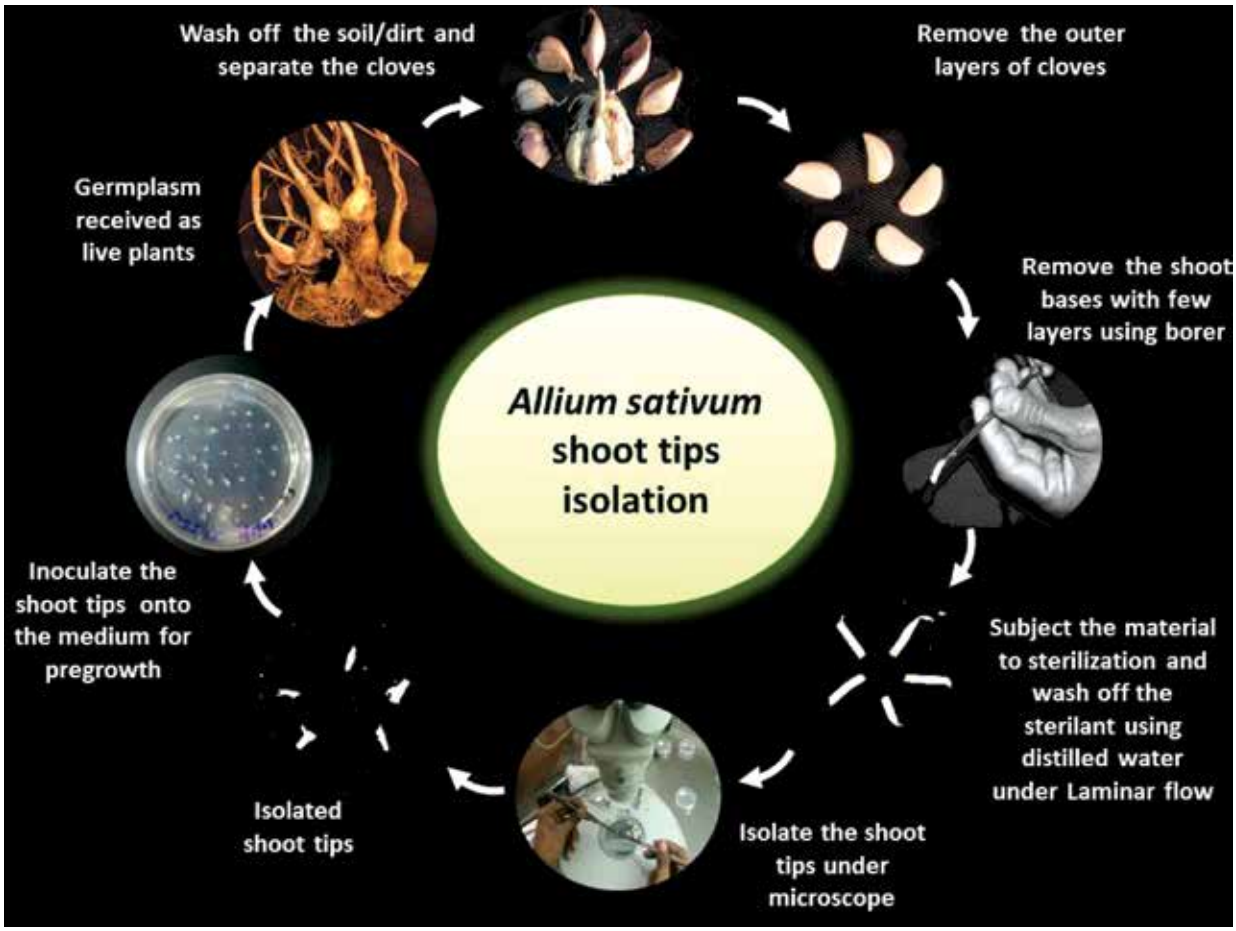


Fig. 1. *Isolation of Allium sativum shoot tips*

e) Thawing and unloading

1. Remove the cryovials with frozen explants and warm in 37°C water bath immediately for 2 min till PVS2/PVS3 became liquid again.
2. Open tubes and remove PVS2/PVS3 solution followed by addition of 1ml unloading solution and hold for 20 min.

f) Regrowth and assessment

1. For regrowth, remove explants from the unloading solution and blot them dry on sterile filter paper, on Petri dish.
2. Transfer explants on to regrowth medium (B5+ 0.1 mg/l NAA + 0.5 mg/l 2iP) in the Petri dishes and keep them in the dark for 3 days in the culture room at 25°C.
3. Transfer the Petri dishes to standard culture room conditions (16 h light/8h dark) at 25°C.
4. Assess the survival, 2 weeks after cryopreservation by greening of the shoot tips and regrowth by counting the number of shoot tips that have developed leaves. Controls refer to shoot tips subjected to all treatments except LN freezing
5. Record the observations everyday up to 4-8 wks.

Droplet vitrification protocol

Follow steps a and b as above.

c) Dehydration with PVS2/PVS3

1. Following pre culture, transfer 10 explants each to a Petri dish (35 mm) containing PVS2 /PVS3 solution.
2. Place aluminum foil strips (30 × 5 mm) on a Petri dish maintained at room temperature and put drops of PVS2/PVS3 on these strips.
3. Two minutes prior to end of the PVS2 (40 min) /PVS3 (120 min) duration, transfer shoot tips from petri dish to the PVS2/PVS3 droplets.

d) Rapid freezing

- Lift the aluminum foil strip holding the shoot tips with a pair of forceps and immerse in LN. After bubbling stops, transfer the strip with droplets into a cryovial (2 ml) kept in a polycarbonate cryobox filled with LN in a Thermocol box of LN.

e) Thawing and unloading

1. Remove the foils strip with the forceps and immerse them in an unloading solution kept in Petri dish, for 20 min at room temperature.

f) Regrowth and assessment

- Follow steps as above.

Note

* *Cryoprotectants and pre culture additives must be of high purity, use of analytical grade sucrose and spectroscopically pure DMSO is recommended.*

* *It is generally recommended to filter sterilize the cryoprotectant mixtures.*

* *Owing to high viscosity of the vitrification solutions, autoclaving is the preferred method of sterilization*

Selected references

Kim HH, E Cho HJ Baek, CY Kim, ERJ Keller and Engelmann F 2004. Cryopreservation of

garlic shoot tips by vitrification: Effects of dehydration, rewarming, unloading and regrowth conditions. *CryoLetters* **25**: 59-70.

Pandey R, A Pandey, N Sharma and KS Negi 2009. Cryopreservation of *in vitro* shoot bases of *Allium tuberosum* (L.) Rottl. *ex* Spreng., by vitrification technique. In: Abstr., 1st International Symposium on "Cryopreservation in Horticultural Species" at Leuven, Belgium, April 5-8, 2009, p113.

Pandey R, N Sharma, and A Agrawal (2018) Cryobanking of Garlic (*Allium sativum* L.) Germplasm at ICAR-NBPGR in India: Achievements, Constraints and Prospects. In: Thammasiri K, P Kongsawadworakul and HW Pritchard (eds), Book of Abstracts, *The Third International Symposium on Plant Cryopreservation (CryoSymp2018)*, Department of Plant Science, Faculty of Science, Mahidol University, Bangkok, Thailand, p 21.

Pandey R, N Sharma, A Pandey, R Gowthami and A Agrawal (2019) *In vitro cryopreservation of underutilized alliums: success and limitations.* In: Abstracts: International Symposium on Edible Alliums: Challenges and opportunities, February 9-12, 2019, Pune, India, p 277-78.

Reed BM 2008. *Plant Cryopreservation: A Practical Guide.* Springer Publication, New York, 511 p.

Sakai A, S Kobayashi and I Oiyama. 1990. Cryopreservation of nucellar cells of navel orange (*Citrus sinensis* Osb. var. *brasiliensis* Tanaka) by vitrification. *Plant Cell Reports* 9: 30–33.

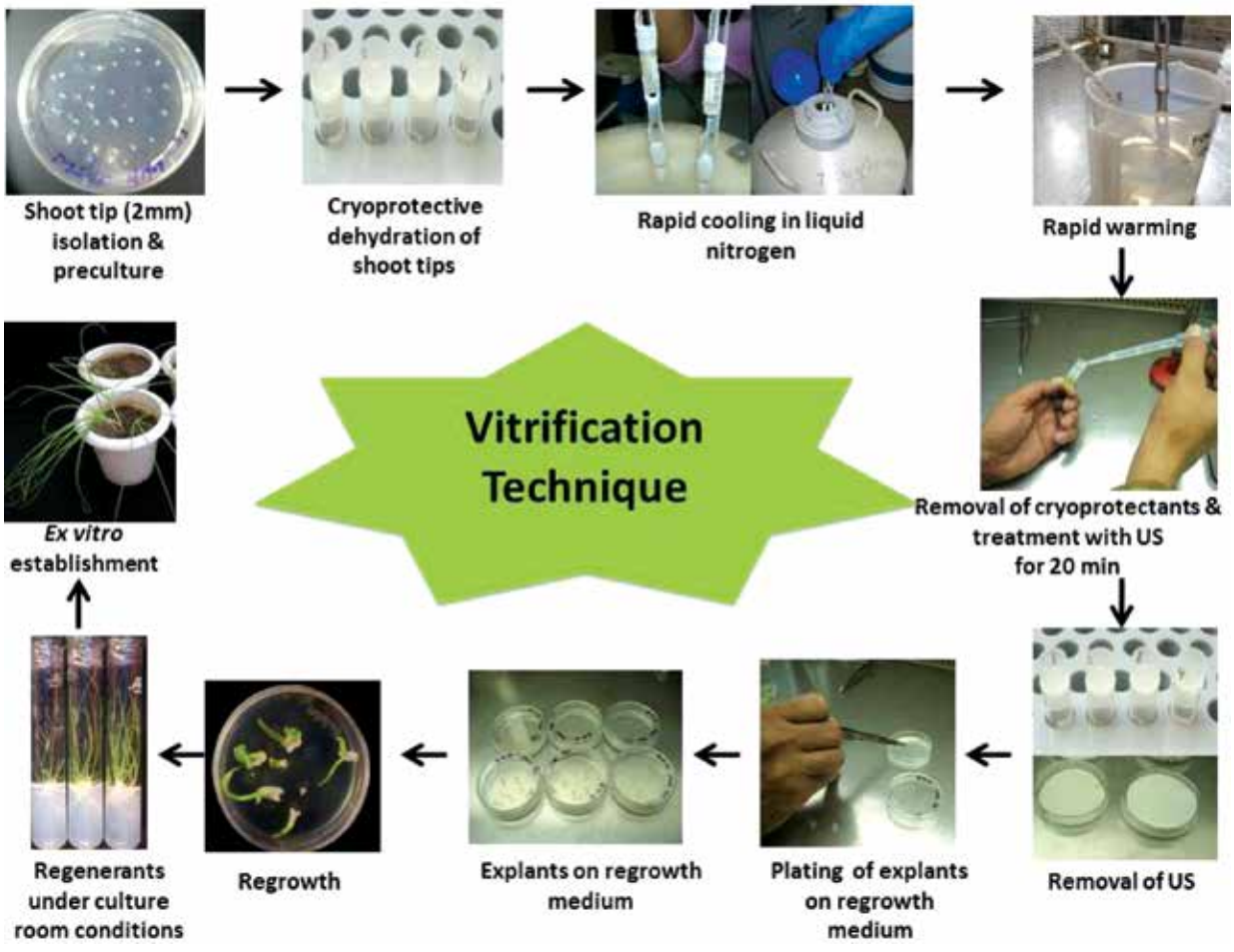


Fig. 2. *Cryopreservation of garlic using vitrification technique*

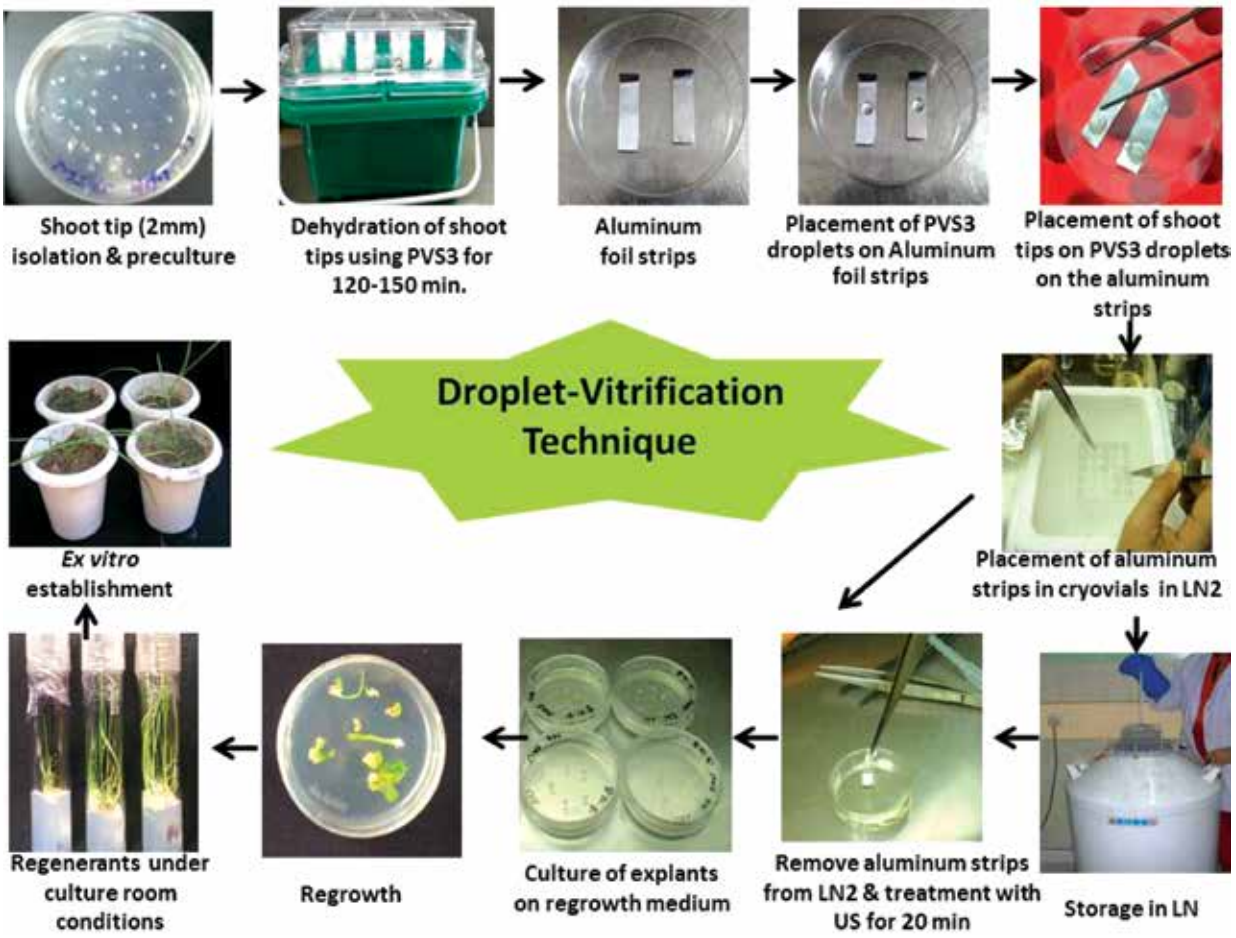


Fig. 3. *Cryopreservation of garlic using droplet vitrification technique*

11. DNA isolation, purification and quantification

Sangita Bansal, Era Vaidya Malhotra, Kanika Sharma, Vikrant, Rishu Jain and Suresh Chand Mali

Introduction

DNA isolation is a process of extraction and purification of DNA from different tissues using a combination of physical and chemical methods. DNA was successfully isolated for the first time by Friedrich Miescher in 1869 (Dahm, 2008). DNA isolation has now become a routine procedure in molecular biology and diagnostics laboratories. Nowadays, a number of different kits, based on chemical extraction methods, are commercially available. Choice of isolation method depends upon a number of factors, including; Source of the DNA (plant leaves, seeds, blood, bacterial cultures, viruses etc.); Type of DNA (chromosomal or extrachromosomal); and Final application (PCR, restriction digestion, sequencing, fingerprinting, library construction etc.)

DNA extraction

There are three basic steps in DNA extraction:

1. Tissue homogenization and cell lysis

Plant tissues are homogenized either mechanically or chemically. Most commonly used method of tissue homogenization is the crushing of samples in liquid nitrogen. Cell lysis is achieved by rupturing the cell membrane or lipid bilayer to extract the DNA along with cell cytoplasm with the help of detergents, namely, sodium dodecyl sulphate (SDS), sarcosine, triton X-100 or cetyl trimethylammonium bromide (CTAB).

2. Removal of impurities

Proteins are either removed by breaking the disulfide bonds using β-mercaptoethanol or they are hydrolysed using proteinases. The solution is treated with high salt concentrations to clump the broken proteins, lipids and polysaccharides together to form debris. Cell debris is removed by centrifugation. Ethylenediaminetetraacetic acid (EDTA) is added to protect DNA by chelating Mg^{++} ions which are essential for nuclease activity. Polyvinylpyrrolidone (PVP) is optionally used to remove polyphenols.

3. Concentration and precipitation of DNA

Phenol : chloroform: isoamyl alcohol extraction is carried out; where phenol denatures proteins, chloroform helps in separation of aqueous and organic phases and phenol removal, while isoamyl alcohol reduces the foaming and stabilizes the interphase. Hydrophobic proteins

and lipids remain in the organic phase while nucleic acids mobilize into the aqueous phase. DNA precipitation is achieved using either isopropanol or absolute ethanol.

Reagents required

1 M Tris (100 ml)

Dissolve 15.764 g Tris HCl in 60 ml distilled water. Adjust the pH to 8.0 and make up the volume to 100 ml. Autoclave and store at room temperature.

0.5 M EDTA (100 ml)

Dissolve 18.60 g EDTA in 60 ml distilled water. Adjust the pH to 8.0 with NaOH pellets and make up the volume to 100 ml. Autoclave and store at room temperature.

10% CTAB (100 ml)

Dissolve 10 g CTAB in 80 ml autoclaved distilled water. Make up the volume to 100 ml.

4 M NaCl (Sodium Chloride) (100 ml)

Dissolve 23.37 g NaCl in 80 ml autoclaved distilled water. Make up the volume to 100 ml.

DNA Extraction Buffer (100 ml)

Stock Solution	Volume to be added	Working Concentration
1 M Tris	10 ml	0.1 M Tris
0.5 M EDTA	4 ml	0.02 M EDTA
1 M NaCl	35 ml	1.4 M NaCl
1 % PVP	1 g	Add freshly
0.2% □- mercaptoethanol	200 µl	Add freshly
10% CTAB	20 ml	2% CTAB

Chloroform : Isoamyl Alcohol (24:1)

Take 96 ml of chloroform and mix with 4 ml isoamyl alcohol. Mix well and store in amber coloured bottle.

70% Ethanol

Mix 70 ml absolute alcohol with 30 ml autoclaved distilled water.

TE Buffer (10 mM Tris, 1mM EDTA)

Take 1 ml of 1 M Tris buffer and 0.2 ml of 0.5 M EDTA and mix with autoclaved distilled water to make up the final volume to 100 ml.

Phenol : Chloroform : Isoamyl Alcohol (25:24:1)

Mix 100 ml of 100 mM Tris saturated phenol with 96 ml chloroform and 4 ml isoamyl alcohol. Mix well and store in amber coloured bottle.

RNase stock (10 mg/ml)

Mix 10 mg of RNase (DNase free) in autoclaved distilled water. Store at -20°C.

Procedure

DNA extraction is carried out following the CTAB – DNA extraction protocol (Doyle and Doyle, 1987). The steps followed for extraction of genomic DNA (Fig. 1) are given below:

1. Weigh 100 mg of the leaf sample, cut into small pieces and grind the frozen leaves into a fine powder in a pre-cooled mortar with the help of a chilled pestle in liquid nitrogen.
2. Suspend the powder into a 2 ml micro-centrifuge tube containing 750 μl of pre-warmed DNA extraction buffer.
3. Mix properly and incubate the tubes in a hot water bath at 65°C for 60 minutes.
4. Add equal volume of chloroform: isoamyl alcohol mixture in the ratio of 24:1. Gently invert the samples for 15 minutes.
5. Centrifuge the mixture for 20 min at 10,000 rpm and collect the aqueous layer carefully into fresh micro-centrifuge tubes.
6. Add 2/3 volume of chilled isopropanol (750 μL) to the aqueous layer, and incubate the tubes overnight at -20°C for DNA precipitation.
7. Next day, centrifuge the samples at 10,000 rpm for 10 min at 4°C to pellet the DNA.
8. Wash the DNA pellet with 70% ethanol and air dry.
9. Dissolve the DNA pellet in 300 μL of TE buffer and store at -20°C.

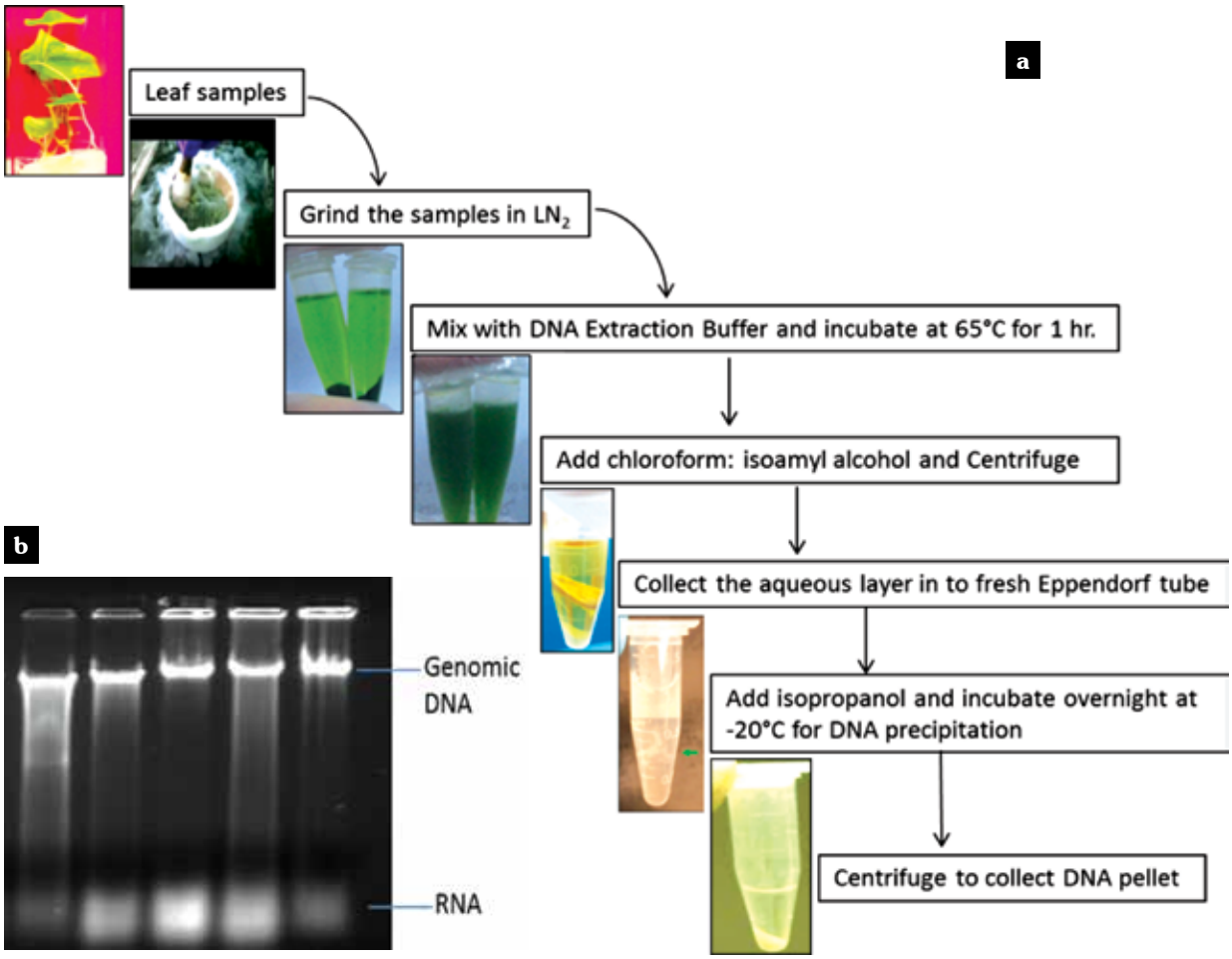


Fig. 1. a) *DNA Extraction Procedure;* ***b)*** *Extracted DNA on 0.8% Agarose Gel*

Purification of genomic DNA

To remove the contamination of RNA and proteins, isolated genomic DNA is further purified by the procedure provided below:

1. Add RNase stock (10 mg/ml) to give final concentration of 10μg/ ml and incubate for 1 hr. at 37°C.
2. Add equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) and mix the contents.
3. Centrifuge the mixture for 20 min at 10,000 rpm and collect the aqueous layer carefully in to fresh microfuge tubes.
4. Add 500 μl of chilled isopropanol and incubate overnight at -20°C to precipitate the DNA.
5. Collect the DNA pellet by centrifugation at 10,000 rpm for 10 min at 4°C.
6. After centrifugation, dry the pellet after washing with 70% ethanol and dissolve in 100 μl of TE buffer, then store at -20°C till further use.

Qualitative and Quantitative Analysis of Isolated DNA

DNA quality is estimated by agarose gel electrophoresis (Fig. 2).

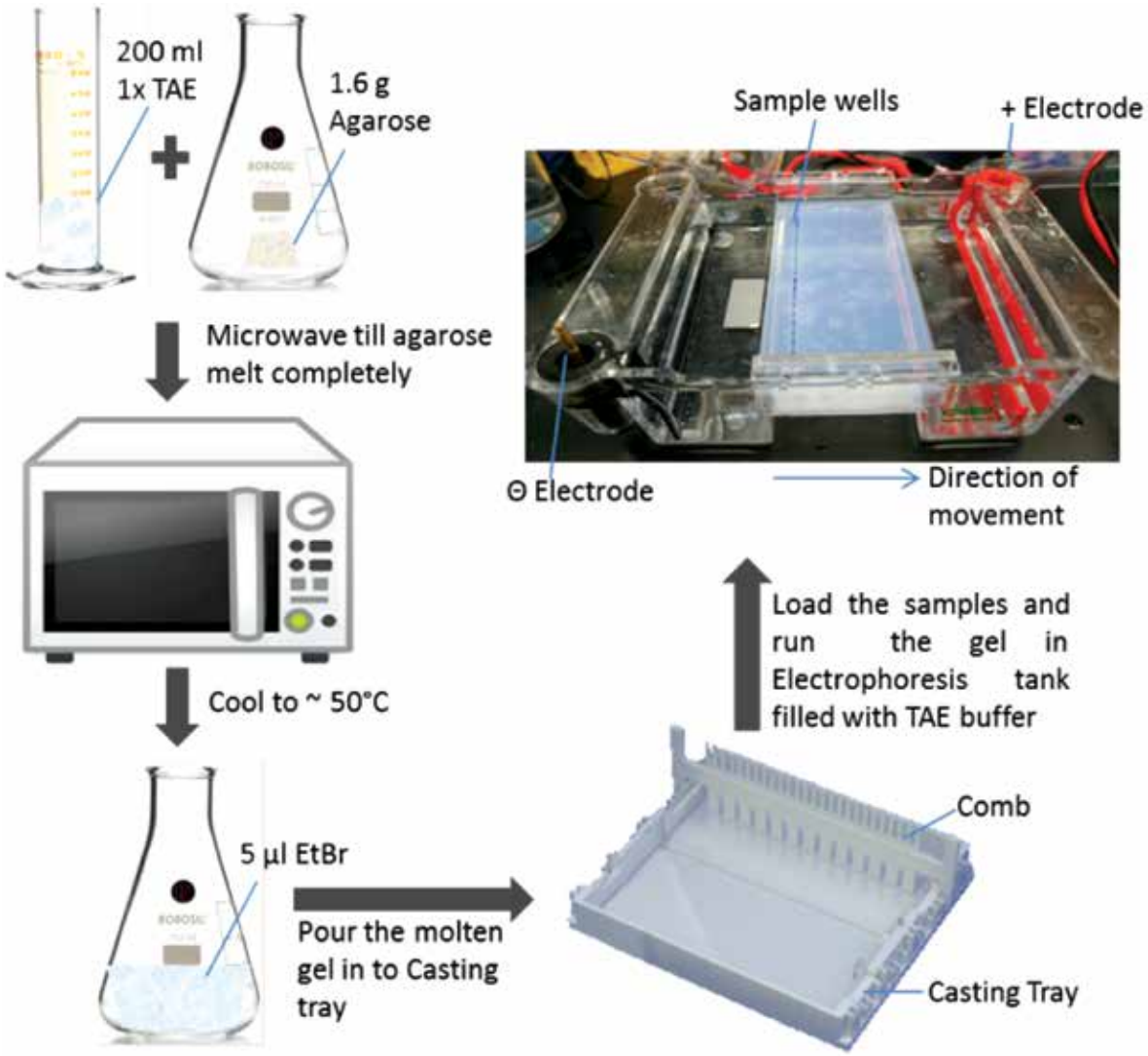


Fig. 2. *Agarose Gel Electrophoresis of Genomic DNA*

Reagents required

1. 50X TAE buffer (100 ml)

- Mix 24.2 g Tris Base, 57.1 ml glacial acetic acid and 100 ml of 0.5 M EDTA (pH 8.0) to make 50X TAE buffer.
- For preparing 1X TAE buffer, dilute 2 ml of 50X TAE buffer with 98 ml distilled water.

2. 6X DNA loading dye (100 ml)

Take 250 mg of Bromophenol blue (0.25%) and 40 g of Sucrose (40%). Dissolve in 100 ml of sterile distilled water.

3. Ethidium Bromide

Dissolve 10 mg of Ethidium Bromide in 1 ml of autoclaved distilled water and store in amber colored bottle.

Procedure

1. Prepare 0.8% agarose gel in 1X TAE buffer. For this, weigh 1.6 g of agarose powder and mix in 200 ml 1X TAE buffer and melt by heating. When the molten gel has cooled, add 5 μl ethidium bromide to it and mix well. Pour the mixture in casting tray with comb to form wells.
2. Mix 10 μl of DNA sample with 2μl of 6X DNA loading dye (0.25% Bromophenol blue, 40% Sucrose).
3. Load the sample on the agarose gel and electrophorese at 80 Volts for 1.5 hr in 1X TAE buffer.
4. Visualise the DNA bands on UV-Transilluminator and take photographs on Gel documentation system.

Quantification of purified genomic DNA can be done either on UV-VIS Spectrophotometer or Nanodrop Microvolume Spectrophotometer. To measure the concentration using UV-VIS Spectrophotometer, set the blank against 100 ml of the TE buffer and then measure OD (optical density, absorbance) of 2 μl DNA sample with 50 times dilution (in TE buffer) at 260 as well as 280 nm. The concentration of DNA is calculated using following equation:

Concentration of DNA (μg/ ml) = OD_{260} × 50 × dilution factor

(1 OD_{260} = 50 μg/ ml of double stranded DNA)

To quantify DNA using Nanodrop Microvolume Spectrophotometer, wipe the optical surfaces of Nanodrop with lint free wipes. Go to the nucleic acid application of Nanodrop software on computer. Place 1 ml of buffer on lower optical surface of Nanodrop, lower the arm and select 'blank' in nucleic acid application. Again clean the optical surfaces and place 1 ml of DNA sample on lower optical surface, lower the arm and select 'measure' in nucleic acid application. Note down the DNA concentration (mg/ml), OD260/280 and OD260/230 ratios to analyze the DNA quality.

The ratio of OD 260 to 280 gives an indication of the amount of RNA or protein contamination in the sample. A value of 1.8 is optimal for the best DNA preparation. A ratio below 1.8 indicates the presence of protein in the sample while a value above 1.8 indicates that sample has RNA contamination. The ratio of OD 260 to 280 for pure DNA ranges from 1.8-2.2.

References

Dahm R (2008) Discovering DNA: Friedrich Miescher and the early years of nucleic acid research. Human Genetics. 122(6): 565–581.

Doyle JJ and JL Doyle (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin*. 19: 11-15.

12. PCR and gel analysis of SSR and ISSR markers for genetic stability studies

Era Vaidya Malhotra, Sangita Bansal, Rishu Jain, Kanika Sharma, Vikrant and Suresh Chand Mali

Introduction

Monitoring and determination of the effect of conservation procedures on the genetic integrity of the material during processing and cryo-conservation is a fundamental requirement of all cryopreservation studies. The aim is to verify that the storage protocol does not have any deleterious effect on the genome due to any sort of cryoinjury or toxicity of the cryoprotectants used during the cryopreservation phase or unorganised tissue growth during recovery and regeneration post cryopreservation. Alterations in the genetic makeup are manifested as detectable changes in morphology, cytology, biochemical parameters and molecular marker profiles to compare the true-to-type nature of plants recovered from cryostorage.

Molecular markers have become the method of choice for researchers to analyse the genetic integrity of conserved germplasm. A number of marker techniques are available for genetic stability analysis, such as, Restriction Fragment Length Polymorphism (RFLP), Amplified Fragment Length Polymorphism (AFLP), Random Amplified Polymorphic DNA (RAPD), Simple Sequence Repeat (SSR), Inter Simple Sequence Repeat (ISSR) and Sequence-related Amplified Polymorphism (SRAP). ISSR and SSR marker systems are most commonly used for these studies, because of their ease of operation. Both of these are Polymerase Chain Reaction (PCR) based approaches.

SSR primers are based on the microsatellite repeats, i.e. DNA stretches of short, tandemly repeated di-, tri-, tetra-, penta- or hexa-nucleotide repeat motifs, flanked by

Fig. 1. *Principle of SSR based molecular markers*

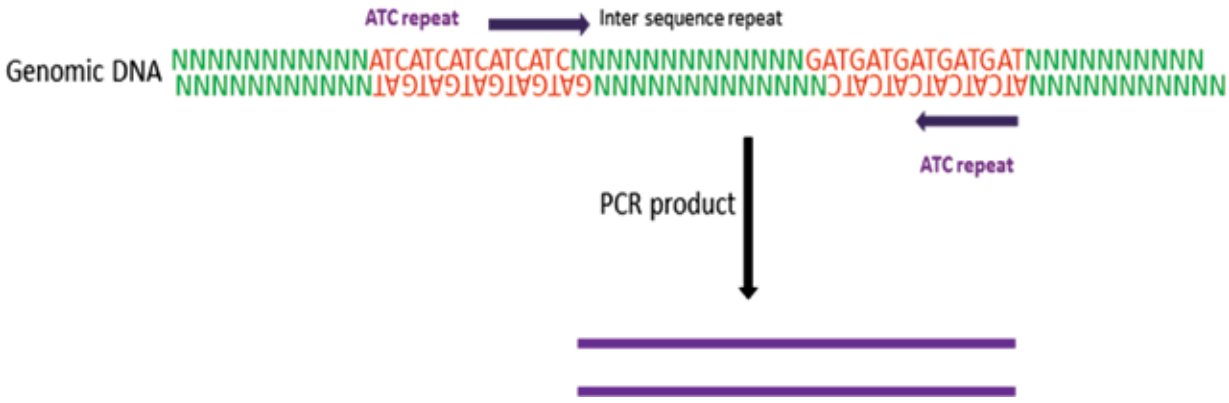


Fig. 2. *Principle of ISSR based molecular markers*

conserved DNA sequences, found in most eukaryotic species (Litt *et al*., 1989). Variation in the samples is reflected as length polymorphisms due to allelic variation of the number of repeat motifs in the microsatellite. In microsatellite analysis, two primers are designed complementary to the sequences flanking a specific microsatellite DNA sequence.

ISSR markers follow the principle of amplification of DNA fragments flanked by inversely oriented SSR motifs (Zietkiewicz *et al*., 1994). Detection of DNA polymorphism depends on the abundance and variability of microsatellite repeats in the genome. This technique uses microsatellite repeat motifs as primers to amplify the inter simple sequence repeats of different sizes. The technique is simple, quick and needs no sequence information for primer synthesis.

Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) is a technique to amplify a DNA fragment to generate millions of its copies in an enzymatic reaction carried out *in vitro*. This technique was invented by Kary B. Mullis in 1983 and he was awarded the Nobel Prize in 1993. PCR is a very simple and inexpensive technique for characterization, analysis and synthesis of specific fragments of DNA or RNA from virtually any living organism.

Principle

PCR is based on the 5' - 3' polymerase activity of DNA polymerase. DNA polymerase can add nucleotides to free 3' end of a DNA fragment in a template dependent manner under standard conditions. Initially, the DNA is denatured to separate out the two strands. To these single strands oligonucleotide primers bind at their complementary sequences. The *Taq* DNA polymerase, extracted from the thermophilic bacterium *Thermus aquaticus*, then begins to add nucleotides to the 3' end of each primer and thereby extends the DNA strand, producing a new daughter strand. This cycle is then repeated a number of times to amplify the number of copies of that particular DNA fragment. This leads to an exponential increase (2^N) in the number of fragments of a particle DNA sequence. The three steps of a PCR cycle are,

Step 1: Denaturation Two strands of the double stranded DNA helix are separated from each other at a temperature of 95°C. The hydrogen bonds between the complementary strands break and this leads to the generation of two single strands of DNA.

Step 2: Annealing The temperature is lowered so that the oligonucleotide primers bind to their complementary sequences in the target DNA.

Step 3: Extension The temperature is raised to 72°C and the DNA polymerase binds to the DNA and extends it in the 5' 3' direction by continuously adding nucleotides.

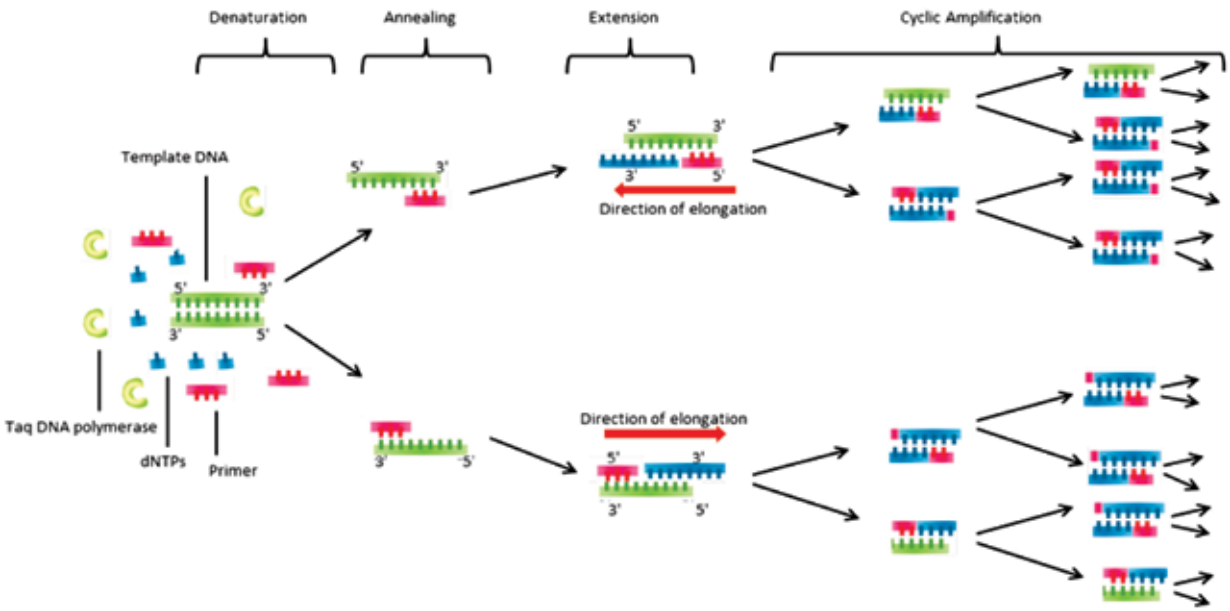


Fig. 3. *Schematic diagram of a PCR*

Components of a PCR reaction

1. DNA Template

The double-stranded DNA segment that is to be amplified is called the DNA template. A trace amount of the DNA template is sufficient.

2. Oligonucleotide primers

These are short single-stranded oligonucleotide sequences (18 – 25 nucleotide long) that are used to amplify a particular known region of a DNA sequence. These are artificially synthesized.

3. Deoxyribonucleotide triphosphate (dNTPs)

Four deoxynucleotide triphosphates, dATP, dCTP, dGTP and dTTP, are required by the polymerase to make new copies of DNA.

4. Divalent Cations

Magnesium ion (Mg^{2+}) is a cofactor required for the activity of the DNA polymerase.

5. Buffer

PCR is carried out in a buffer that provides a suitable chemical environment for the activity of DNA polymerase. The buffer pH is usually between 8.0 and 9.5 and is often stabilized by Tris-HCl.

6. DNA Polymerase

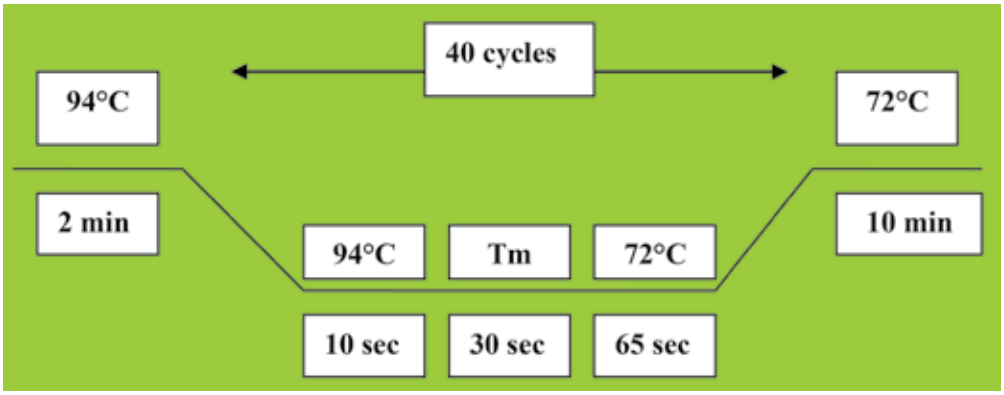
A thermostable DNA polymerase enzyme is required for synthesis of new strands of DNA, complementary to the target sequence, to generate multiple copies.

Table 1. PCR reaction mix composition and concentrations of components used

Components	Volume for a 20µl reaction	Final concentration
PCR buffer containing $MgCl_2$	2 µl	1 X
dNTPs (1mM)	5 µl	250 µM each
Primer (10 µM)	1µl	1 µM
Taq DNA polymerase (5U)	0.2 µl	1.0 U
Template DNA	2 µl	40ng
Autoclaved Distilled Water	To make up the final volume 20µl	

Table 2. General PCR thermal profile

Steps	Temperature	Duration
1. Initial denaturation	94°C	2 minutes
2. 40 cycles each of		
i) Denaturation	94°C	10 seconds
ii) Annealing	As per primer	30 seconds
iii) Extension	72°C	65 seconds
3. Final Extension	72°C	10 minutes
4. Hold at 4°C		



Procedure

1. Allow the components to thaw at room temperature.
2. Set up the reaction in a 2ml microfuge tube. Add all the components in the desired volumes except DNA.
3. Spin the master mix for 10 seconds.
4. Dispense 18µl of master mix in nine different tubes.
5. Add 2µl DNA in each tube.
6. Place the tubes in a thermal cycler and start the reaction.
7. Remove tubes from the machine after the reaction is over and proceed for electrophoresis.
8. Prepare 180 ml of 2% agarose solution gel.

9. Heat the solution after dissolving agarose till it dissolved completely.
10. Allow it to cool and add 4μl of ethidium bromide into the beaker.
11. Cast the gel and allow it to solidify.
12. To run, gently remove the comb, place the tray in electrophoresis chamber and submerged into electrophoresis buffer.
13. Mix 2μl of 6X bromophenol blue in each PCR tube containing amplified DNA.
14. Load 6 μl of the PCR product per well of the gel.
15. Load 3μl 100bp ladder into the first well.
16. Connect the electrodes to the power pack and carry out electrophoresis at 80 volts until the gel is reached three-fourth of gel.
17. Examine the gel under UV light in a UV trans illuminator and photographed in a gel documentation system.

Gel scoring and result interpretation

The amplification products of a PCR reaction are visualized and photographed under UV light. The amplification products are scored by comparing their respective migration distances across the gel lanes, accession wise. Presence of a band is recorded as '1' and absence as '0', while missing data is recorded as '9'. Data is recorded for the number of primers screened, total number of bands observed per primer and number of polymorphic as well as monomorphic primers.

The similarity between the tested samples is computed by calculating the similarity coefficients for pairwise comparisons using softwares, such as NTSYSpc (Rohlf, 1998).

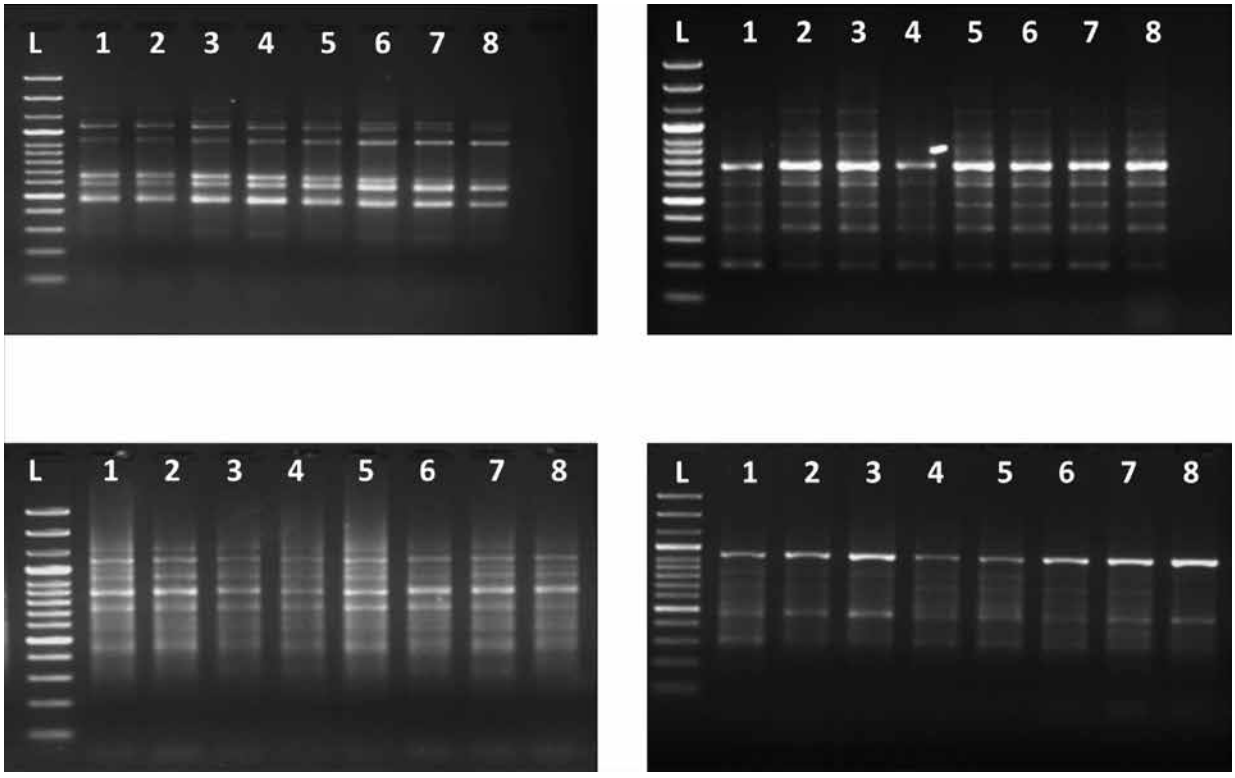


Fig. 4. *PCR amplification of cardamom DNA using two ISSR primers:* ***(a)*** *Amplification profile of Primer UBC 813;* ***(b)*** *Amplification profile of Primer UBC 825; L – molecular weight marker; 1: Mother plant; 2 – 8: Micropropagated plants*

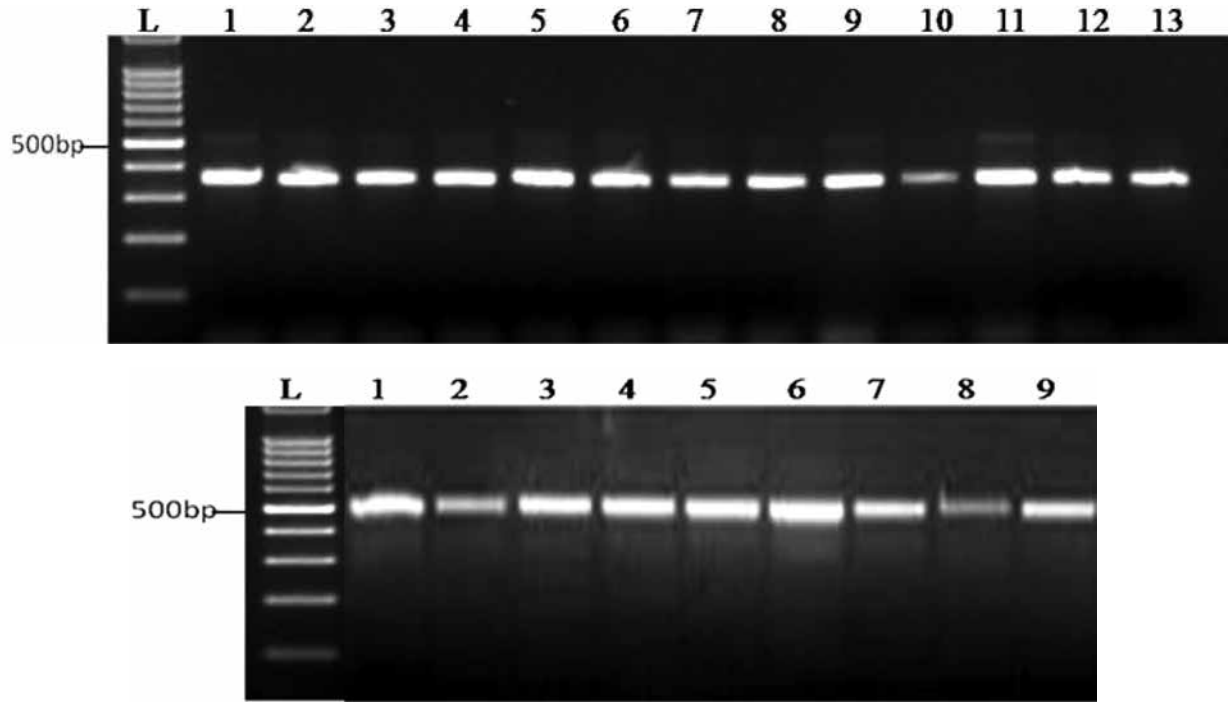


Fig. 5. *PCR amplification of Musa DNA using two EST - SSR primers:* ***(a)*** *Amplification profile of Primer E-P5;* ***(b)*** *Amplification profile of Primer E-P6; L – molecular weight marker; 1: Mother plant; 2 – 13: Micropropagated plants*

Selected references

Litt M, J Luty, A Am and J Hum (1989) A hypervariable microsatellite revealed by in vitro amplification of a dinucleotide repeat within cardiac muscle actin gene. *Am. J. Hum. Genet* 44: 397–401.

Rohlf FJ (1998) NTSYS-pc Numerical Taxonomy and Multivariate Analysis. Version 2.0. Applied Biostatistics Inc., New York, USA. 37p.

Zietkiewicz E, A Rafalski and D Labuda (1994) Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. *Genomics* 20: 176–183.

13. Virus Testing of *in vitro* Germplasm

V. Celia Chalam, Vaishali Dutta Sharma and A.K. Maurya

Introduction

Germplasm material is multiplied, exchanged and conserved either as true seeds, vegetative propagules (such as bulbs, rhizomes, suckers, rooted cuttings, etc.) or as *in vitro* cultures. Since both seeds and vegetative propagules are efficient carriers of viruses, infection is progressively transmitted through generations. Further, if the *in vitro* cultures are raised from a virus infected mother plant, the plantlets are bound to carry the viral infection. The strategy to be employed for testing plant germplasm would not only depend on the nature of material to be indexed but also on the purpose for which testing is to be done.

In case of *in-vitro* cultures it is always advisable to ensure that the mother plant used for raising cultures is free from all the viruses that can affect that plant species. During routine multiplication/propagation using meristem tip culture technique, plants should always be tested against known viruses infecting that crop to ensure that they are virus-free.

A number of physical, serological and molecular techniques are available for detecting plant viruses. These techniques are adopted either alone or in combination, based on their availability and need. These are enumerated below in brief:

Electron microscopy

The Transmission Electron Microscope (TEM) can be used directly to detect the presence of virus in the plant tissue. It reveals the shape and size of the virus particle. The shape and size of the virus particle gives an idea of the group to which it may belong.

The protocol for leaf dip preparation is given below:

1. Cut a small portion of leaf (about 2.0 mm) with the help of a sterilized sharp blade. Grind the sample over a clean glass slide in 2-3 drops of 0.07 M phosphate buffer (pH 6.5).
2. Place 10μl of sample extract on parafilm.
3. Place the carbon coated (darker) surface of the copper grid over droplet, ensuring that the grid surface becomes wet.
4. After one minute, pick up the grid by its edge with fine forceps and wash the grid with continuous flow of 10-12 drops of double distilled water (DDW) to remove the sap.
5. Carefully add 2-4 drops of 2% uranyl acetate (UA) on the filmed surface of the grid.

6. Remove excess stain with a strip of filter paper. Allow the grid to dry.
7. Observe the grid under Transmission Electron Microscope (TEM) for virus particles (Fig. 1).

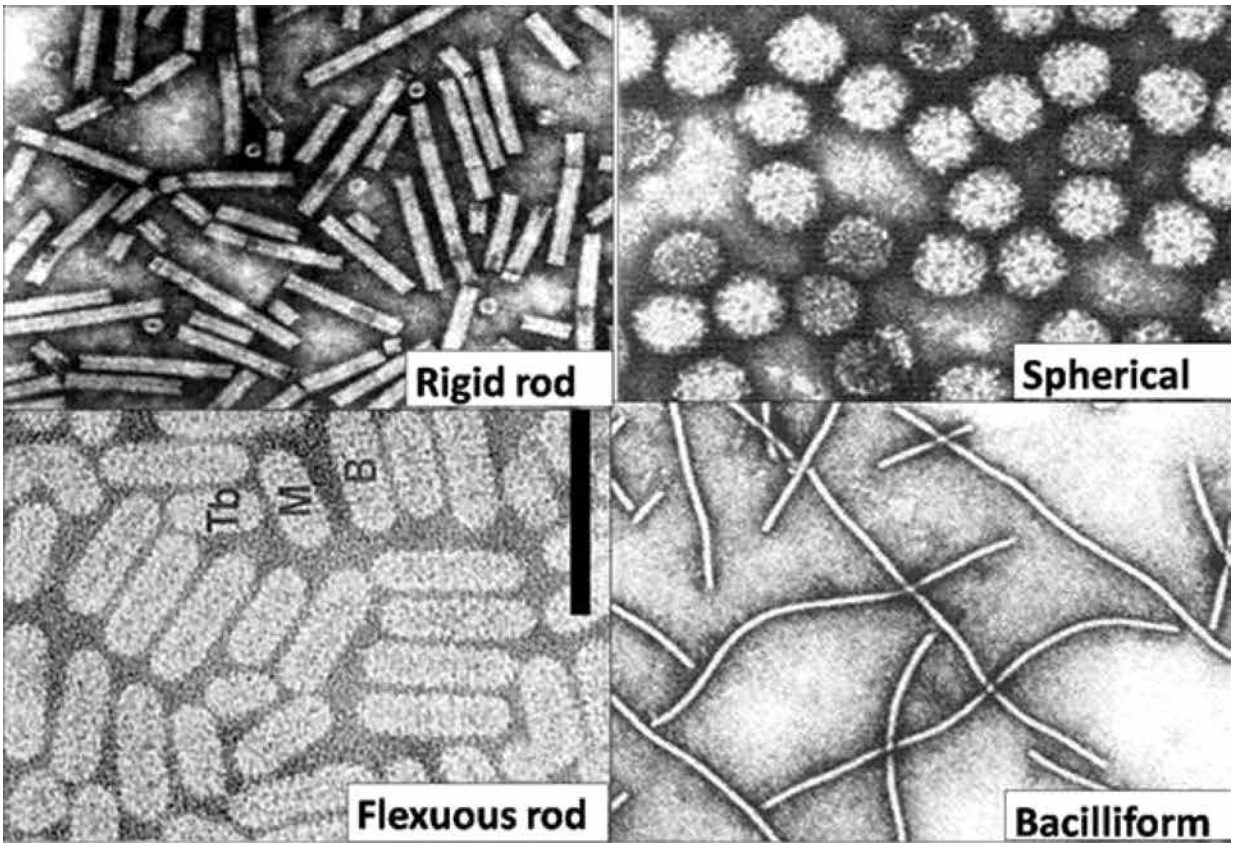


Fig. 1. *Different virus particles visualized under Transmission Electron Microscope*

Double Antibody Sandwich–Enzyme-linked Immunosorbent Assay (DAS-ELISA)

The double-antibody sandwich (DAS)-ELISA is a direct ELISA and was the first ELISA procedure developed for plant virus detection. It is still widely used and many viruses have been detected using ELISA (Chalam and Khetarpal, 2008; Chalam *et al.*, 2017; Chalam and Maurya, 2018).

The protocol is given below:

1. Take 0.1g of leaf sample in a mortar and grind in Phosphate Buffer Saline - Tween 20 (PBS-T) buffer, pH 7.4 + 2% polyvinyl pyrrolidone.
2. Centrifuge the extracts (1:10 w:v) for 3 min. at 3000 rpm and collect the supernatant.
3. Further process the samples by DAS-ELISA.

Buffers used for DAS-ELISA

Carbonate Buffer (pH, 9.6)

Na_2CO_3	1.59 g
$NaHCO_3$	2.93 g

Dissolve in distilled water and dilute to make 1L

Washing Buffer - Phosphate Buffer Saline - Tween 20 (PBS-T), pH 7.4

NaCl	8.0 g
Na_2HPO_4 12 H_2O	2.9 g
KH_2PO_4	0.2 g
KCl	0.2 g
Tween 20	0.5 ml

Dissolve in distilled water and dilute to make 1L

Sample Extraction Buffer, pH 7.4

Polyvinyl pyrrolidone	20 g
NaN_3	0.1g

Dissolve in PBST and dilute to make 1L

Conjugate Buffer, pH 7.4

Polyvinyl pyrrolidone	20 g
Bovine serum albumin	2 g
NaN_3	0.1 g

Dissolve in PBST and dilute to make 1L

Substrate Buffer, pH 9.8

Diethanolamine	97 ml

Dissolve in 800 ml distilled water and adjust pH to 9.8 with 1N HCl, adjust the volume to 1L with distilled water.

Substrate Solution

Para-nitro phenyl phosphate (pNPP)1 mg/ ml in substrate buffer

Yellow colour is observed in samples infected by virus. Consider the sample as infected if its OD value is twice the mean of negative control (Fig. 2).

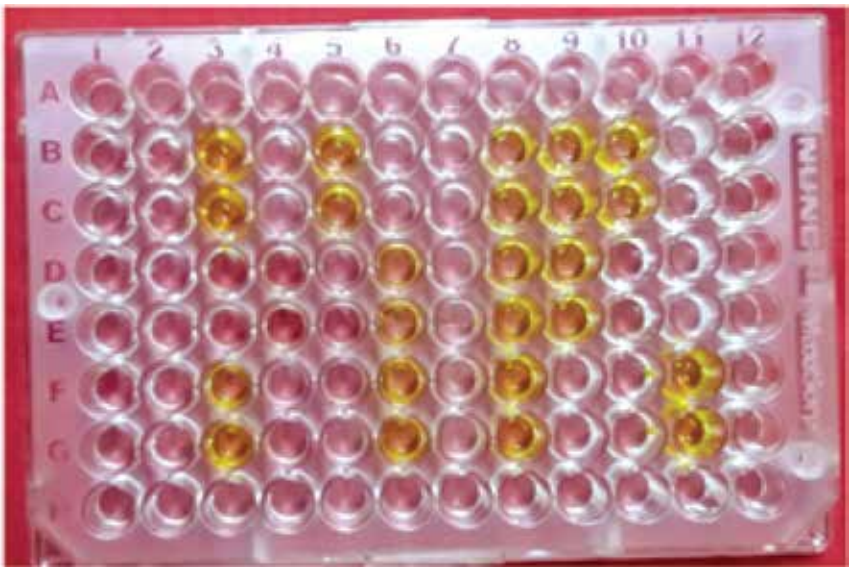


Fig. 2. *DAS-ELISA of yam against Dioscorea latent virus*

Protocol Followed for DAS-ELISA

Deposit the coating antibodies to a specific virus diluted 1\100 or as required in carbonate buffer, pH 9.6 (100 µl/ well) in microtitre plate and incubate for 3 to 4 hrs at 37°C.

↓

Discard the reagents quickly and wash the plate three times with washing buffer PBS –T pH 7.4 at an interval of 3 min.

↓

Deposit the sample (each sample in duplicate wells @ 100 µl/ well) and incubate at 4°C overnight.

↓

Discard the reagents quickly and wash the plate three times with washing buffer PBS-T pH 7.4 at an interval of 3 min.

↓

Deposit the antibodies to a specific virus conjugated with alkaline phosphatase (diluted 1/200 or as required in conjugate buffer, pH 7.4) @ 100 µl/ well and incubate for 3 to 4 hrs at 37°C.

↓

Discard the reagents quickly and wash the plate three times with washing buffer PBS-T pH 7.4 at an interval of 3 min.

↓

Deposit the substrate solution @ 100 µl/ well and incubate at room temperature (28±2°C).

↓

Record the optical density (OD) values at 405 nm using ELISA reader after 15 min., 30 min., 1hr and 2 hrs after incubation.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Most of the plant viruses consists of RNA, which require the introduction of a preliminary reverse transcription (RT) step before the PCR amplification process (RT-PCR), thus allowing the amplification of RNA sequences in a cDNA form. Many viruses have been detected using RT-PCR (Liu *et al.*, 2012; Chalam *et al.*, 2017; Chalam and Maurya, 2018).

The procedure for RT-PCR is given below:

RNA extraction

The total RNA isolation from the ELISA positive BCMV infected leaf samples and healthy leaf samples (ELISA negative) using RNeasy® Plant Mini Kit (QUIAGEN® kit catalogue Nos.74903 and 74904).

1. Weigh 100 mg of the infected and healthy leaf tissue.
2. Immediately place the leaf tissue in DEPC water treated pestle and mortar and grind into fine powder by liquid nitrogen and transfer to RNAse free, 2 µl microcentrifuge tube.
3. Add 450 µl Buffer RLT to the leaf powder and vortex vigorously.

4. Transfer lysate to QIAshredder spin column (lilac) placed in 2 ml collection tube. Centrifuge for 2 min at full speed.
5. Transfer the supernatant of the flow-through to a new micro centrifuge tube without disturbing the cell-debris pellet.
6. Add 0.5 volume of ethanol (100%) to the cleared lysate, and immediately mix by pipetting.
7. Transfer 650 µl samples to RNeasy Mini spin column (pink) in a 2 ml collection tube.
8. Centrifuge for 15s at 10,000 rpm. Discard flow through.
9. Add 700 µl Buffer RW1 to the RNeasy spin column.
10. Centrifuge for 15s at 10000 rpm and discard flow through.
11. Add 500 µl Buffer RPE to the RNeasy spin column.
12. Centrifuge for 15s at 10000 rpm. Discard flow through.
13. Add 500 µl Buffer RPE to the RNeasy spin column. Centrifuge for 2 min at 10000 rpm.
14. Place RNeasy spin column in a new 1.5 ml collection tube.
15. Directly add 30-50 µl TE buffer to the spin column membrane.
16. Centrifuge for 1 min at 10000 rpm to elute the RNA.

Synthesis of cDNA

Use Thermo Scientific Verso cDNA Synthesis kit

1. Prepare 20 µl reaction mixture by adding the ingredients such as 4 µl 5X cDNA synthesis buffer, 2 µl dNTP mix, 1 µl Random hexamer RNA primer, 1 µl RT enhancer, 1 µl Verso enzyme mix, 4 µl viral RNA (template RNA) and use nuclease-free water finally to make up the volume.
2. Reverse transcribe the mixture at 42°C for 30 min and inactivate at 95°C for 2 min.
3. The cDNA thus obtained to be used for standardization of PCR.

PCR

The cDNA synthesized is amplified by PCR using primers specific to each virus and after optimizing annealing temperatures.

1. Conduct PCR amplifications in thermocycler in 20 µl reaction mixture containing 5 µl template cDNA, 10 µl Go Taq® Master Mix 2X (Promega, Madison WI USA), 1 µl each of 5 µM forward and reverse primers and nuclease-free water to make up the volume.
2. Carry out the amplification as follows in thermal cycler:

Cycle	Denaturation (Temp./ Time)	Annealing (Temp./ Time)	Extension (Temp./ Time)
First cycle	94°C/ 4 minutes	-	-
Second cycle	94°C/ 30 seconds	50°C/ 30 seconds (vary according to the amplicon size)	72°C/ 45 seconds
Third cycle	-	-	72°C/ 7 minutes

3. Analyse the amplified products by agarose gel electrophoresis.

Analysis of RT -PCR product by agarose gel electrophoresis

Prepare 1.2% agarose gel by dissolving agarose in 1X TAE buffer.

4. Add ethidium bromide to the agarose at the rate of 0.5 µg/ml of agarose.
5. Add 1 µl of loading dye to the 5 µl of each PCR product.
6. Load samples into wells of the gel along with 100 bp DNA ladder (Thermo Scientific GeneRuler) and run at 60 V for 1hr 15 min.
7. Remove gel from the casting tray, visualize under UV and photograph gel documentation unit.

A band of the DNA fragment at expected base pair length is observed indicating amplification. Its molecular weight is determined by comparing with the molecular weight of marker run on the same gel (Fig. 3).

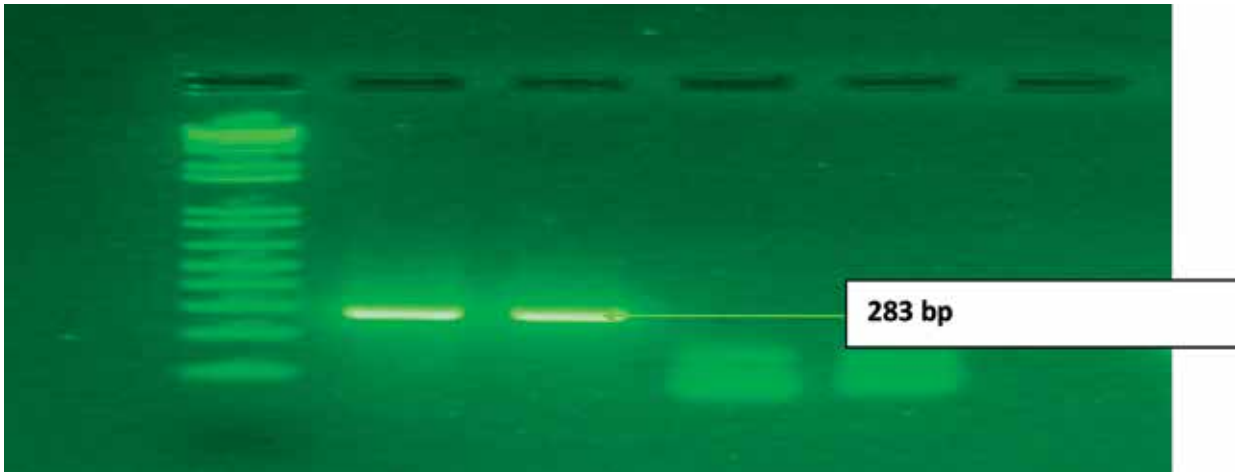


Fig. 3. *Gel Electrophoretic Analysis of RT-PCR of Cherry leaf roll virus*

Viruses detected in *in vitro* cultures at ICAR-NBPGR

At ICAR-NBPGR, viruses have been detected in germplasm of *in vitro* cultures of *Allium* pp., *Fragaria* spp., *Musa* spp., and *Dioscorea* spp. The important viruses include *Carnation latent virus*, *Garlic common latent virus*, *Onion yellow dwarf virus* and *Irish yellow spot virus* in *A. sativum*; *Arabis mosaic virus*, *Raspberry bushy dwarf virus*, *Raspberry ringspot virus*, *Strawberry latent ringspot virus*, *Strawberry mild yellow edge virus*, and *Tobacco streak virus* in *Fragaria* spp., *Dioscoria latent virus* and *Dioscorea mosaic virus* in *Dioscorea* spp. and *Banana bract mosaic virus* in *Musa* spp. (Parakh *et al.*, 2016).

Conclusion

The techniques for rapid, specific and sensitive detection of plant pathogenic viruses have improved in terms of quality and variety during the last few years. To the extent possible, the new technologies should be integrated with conventional tools now in use, so as to complement but not to substitute the latter.

Suggested reading

Bhalla S, VC Chalam, A Lal and RK Khetarpal (2009) *Practical Manual on Plant Quarantine*. National Bureau of Plant Genetic Resources, New Delhi, India p. 204 + viii.

Boonham N, J Kreuze, S Winter, René van der Vlugt, J Bergervoet, J Tomlinson and J Mumford (2014) Methods in virus diagnostics: From ELISA to next generation sequencing. *Virus Res*. 186: 20-31.

Chalam VC, K Gupta, S Bhalla, Rajan and RK Khetarpal (2009) *Training Manual on Biosecurity and Biosafety: Policies, Procedures and Issues.* National Bureau of Plant Genetic Resources, New Delhi, India, 392p.

Chalam VC, DB Parakh and AK Maurya (2017) Role of viral diagnostics in quarantine for plant genetic resources and preparedness. *Indian Journal of Plant Genetic Resources* 30 (3): 271-285.

Chalam VC and AK Maurya (2018) Role of quarantine in ensuring biosecurity against transboundary plant viruses. *Agric. Res. J.* 55 (4): 612-626.

Clark MF and AN Adams (1977) Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. *J. General Virol.* 34: 475-483.

Khetarpal RK, DB Parakh and VC Chalam (2003) Virus indexing of plant germplasm. In: *Conservation biotechnology of plant germplasm.* National Bureau of Plant Genetic Resources, New Delhi, India/ IPGRI, Rome, Italy/ FAO, Rome Italy, pp 99-104.

Liu F, L Feng, X Chen, Y Han, W Li, W Xu, B Cai and M Lin (2012) Simultaneous detection of four banana viruses by multiplex PCR. *Journal of Phytopathology* 160: 622-627.

Parakh DB, VC Chalam, S Gupta, AK Maurya, R Yadav and D Sharma (2016) Virus-indexing of *In Vitro* Conserved Strawberry Germplasm in *In Vitro Gene Bank*, India. pp 92 In: S Archak, Agrawal A, JC Rana, JL Karihaloo and RK Tyagi (eds.) *Abstracts Book. 1st International Agrobiodiversity Congress,* November 6-9, 2016, New Delhi, India, 344 p.

Notes

Notes

Notes

Notes

Notes