Management of Citrus Genetic Resources

SOPs for Collection and Conservation

SK Malik
S Bansal
RK Kakoti
J Uchoi
SP Ahlawat

ICAR-National Bureau of Plant Genetic Resources
Pusa Campus, New Delhi-110 012 India

Azadi Ka Amrit Mahotsav

भा.कृ.अनु.प.
ICAR

रा.पा.आ.सं.ब्यूरो
NBPGR

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DBT Sponsored Project:
Collection, Conservation and Morpho-phenological Characterization of Citrus Germplasm of North East Region

SK Malik
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RK Kakoti
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SP Ahlawat

ICAR-National Bureau of Plant Genetic Resources
Pusa Campus, New Delhi-110 012 India

The ICAR-**National Bureau of Plant Genetic Resources** (ICAR-NBPGR), is a nodal organization under the aegis of Indian Council of Agricultural Research (ICAR) for the management of plant genetic resources in India. ICAR-NBPGR operates as per the mandate of the Government of India and actively contributes to global efforts in ensuring food and nutritional security. The institute also recognizes the need to integrate *ex situ* and *in situ* conservation approaches in a network mode with all its stakeholders. ICAR-NBPGR's mission is to ensure the country's agricultural growth and development by ensuring unrestricted availability of germplasm and associated information for use in research and utilization as per the national and international legislations.

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भारतीय कृषि अनुसंधान परिषद्
डॉ राजेंद्र प्रसाद रोड, कृषि भवन, नई दिल्ली-110001
Indian Council of Agricultural Research
Dr. Rajendra Prasad Road, Krishi Bhavan, New Delhi-110001
फोन/कार्यालय Tel (Off.): 011 23382257, 23046457; E-mail: adgseed.icar@nic.in

डॉ. डी.के. यादव
सहायक महानिदेशक (बीज)
Dr. D. K. Yadava
Assistant Director General (Seed)

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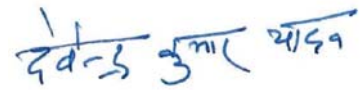
Foreword

India is endowed with vast genetic resources of horticultural crops with remarkable diversity existing in fruits and vegetables. Southeast Asia is represented by more than 500 species of fruits, while the Hindustani region of diversity represents 344 species of fruits. Management of horticultural genetic resources especially fruits is critical being perennial trees, clonally propagated and producing difficult-to-store propagules. Citrus is one of the most economically important fruit crops of India after mango and banana. Diversity of Citrus is extensively distributed throughout the tropical and subtropical regions of the world and India has an enormous variability in both cultivated and wild genetic resources especially concentrated in northeastern and northwestern region. Perceiving vast utility of these resources in citrus industry and crop improvement programmes, efforts were made for understanding the diversity and concomitant safe conservation based on both conventional and biotechnological methods. Several in situ and ex situ methods have been applied to conserve these genetic resources by various Indian institutes. Various State Agricultural Universities, State Horticulture Departments and ICAR institutes have played pivotal role during last five decades in ex situ conservation of Citrus genetic resources by maintaining field genebanks, and also facilitating enrichment of germplasm in cryogenebank at ICAR-NBPGR, New Delhi. Germplasm maintained in the field genebanks always face a risk of losses due to biotic and abiotic factors and also demand high expenditures along with land resources. Therefore, cryopreservation of germplasm of woody species is preferred for long-term conservation and involves the maintenance of explants in aseptic conditions with easy to exchange the germplasm.

There are several protocols and procedures being developed in the laboratories which are published as research papers and manuals. However, to achieve quality results and to carry out the procedures unambiguously and correctly Standard Operating Procedures (SOPs) have great importance. In case of experimenting and handling biological system it becomes rather critical to achieve similar results while applying routine methods, however, following SOPs available at work place it makes easy to achieve repeatability of results.

ICAR-National Bureau of Plant Genetic Resources being pioneer in collection and conservation especially cryopreservation of plant germplasm has developed excellent skills in these areas during last 2-3 decades. I am delighted to know that Tissue Culture and Cryopreservation Unit of ICAR-NBPGR has developed Standard Operating Procedures (SOPs) for the collection and conservation of Citrus genetic resources. I appreciate the well experienced authorship who have been working on management of Citrus genetic resources since last several years and now brought out these SOPs for the benefit of upcoming scientists and students who wish to take up this work routinely. I am happy to know that DBT sponsored project on "Collection, Conservation and Morpho-Phenological Characterization of Citrus Germplasm of North East Region" being implemented at HUB (AAU-Citrus Research Station, Tinsukia), Mirror sites (ICAR-National Bureau of Plant Genetic Resources, New Delhi and Regional Station, Umiam and ICAR-Central Citrus Research Institute, Nagpur) and at various spoke centres in NE States would contribute significantly in managing Citrus Genetic Resources of NE India.

I compliment the authors for bringing out these SOPs as a publication "Management of Citrus Genetic Resources: SOPs for Collection and Conservation".



(D.K. Yadava)
ADG (Seed) and ADG (NASF)

Preface

The present publication aims to provide comprehensive standard procedures for collection and conservation of citrus germplasm. In India, citrus is the third important fruit crop after mango and banana and is distributed throughout the country in diverse climatic regions from tropics to temperate. Only few selected *Citrus* species are being commercially cultivated and rest are occurring in wild or semi-wild forms. There is an urgent need to protect and conserve this genetic diversity for posterity and utilization. Being a diverse fruit crop integrated approach including *in situ* and *ex situ* strategies is recommended for conservation of citrus genetic resources. Among *ex situ*, field gene banks are most common mode of conservation for *Citrus* species, however, these are susceptible to different biotic and abiotic threats and maintenance is cost intensive. In the recent years cryopreservation of plant genetic resources has been largely applied as a method for long-term conservation being economical, climate resilient, requiring less space and eliminating need of regeneration at regular intervals. During last several years exploration, collection and cryopreservation of Citrus germplasm of India is being undertaken as a mission at ICAR-NBPGR and several collections have been made from diverse sources. Protocols and practices have been developed for cryopreservation and successful cryobanking of collected citrus germplasm. Based on our practical experiences we have compiled complete stepwise procedures involved in collection, field genebank and cryopreservation of citrus germplasm in the form of standard operating procedures (SOPs). Illustrations and photographs have been provided for easy understanding. We believe these SOPs would serve as a comprehensive resource for all researchers and laboratories involved in the management of germplasm of citrus and other fruit crops.

We wish to thank the Director, ICAR-National Bureau of Plant Genetic Resources, New Delhi; OIC, Tissue Culture and Cryopreservation Unit (TCCU) and Head, Division of Germplasm Exploration and Collection (DGEC) for valuable guidance and support. We would like to acknowledge the great efforts of scientists and students of cryolab of TCCU and DGEC especially Dr. Rekha Chaudhury, Dr. Rajwant Kalia, Dr. Susheel Kumar, Dr. Ravish Chaudhary, Dr. Digvender Pal, Dr. Rohini MR, Dr. Ajit Uchoi, Dr. Nemappa Lambani, Dr. Sukhdeep Kaur, Mr. O. P. Dhariwal, Mr. Anang Pal Singh, Ms Alka Shukla, Mr. Manuj Avasthi, Mr. Kiran Kumar Murasing and all others who directly or indirectly helped in Citrus collection, cryobanking and also in publication of this document.

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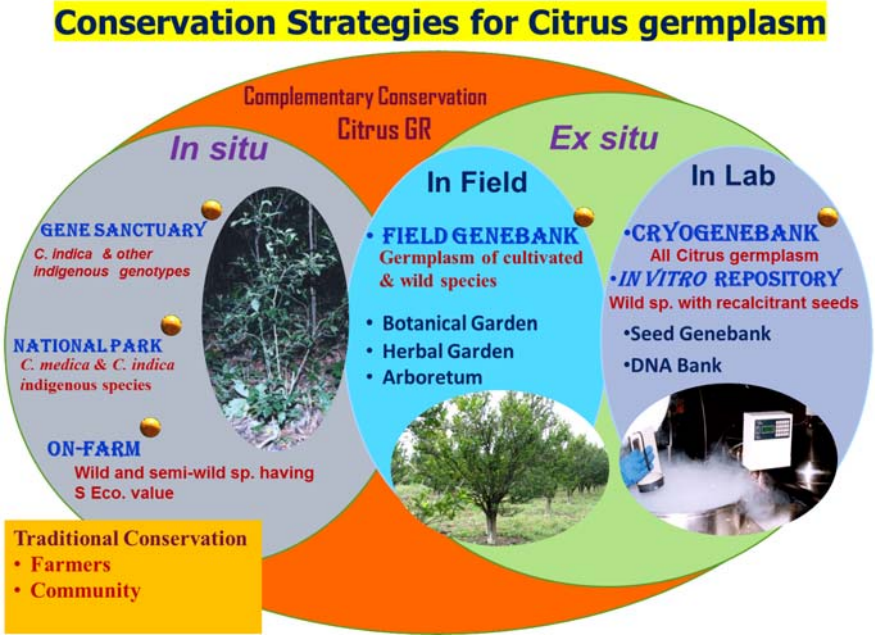
Authors

CONTENT

1. Introduction

Citrus is one of the most important fruit crops of India with vast genetic resources existing both in cultivated as well as wild species. Enormous genetic diversity available in northeastern and other parts of India requires proper management for long-term conservation and utilization for crop improvement. Several attempts have been made to collect and conserve this valuable genetic diversity in India since long. Efforts are being made to conserve this important germplasm in the field gene banks by several horticultural institutions, State Agricultural Universities and State Horticulture Departments. However, conservation of Citrus germplasm in the Field Gene banks (FGB) has several problems including vulnerability to climate change. Due to these issues field gene bank conservation of Citrus germplasm is posing serious challenge and solution only lies in adopting complimentary conservation strategies where both *in situ* and *ex situ* conservation are to be attempted. Most of the Citrus species being polyembryonic (having both zygotic and nucellar embryos in seeds) provides excellent opportunity to conserve the germplasm using seeds/embryos/embryonic axes. This would serve the purpose of genotypic as well as genetic diversity conservation of cultivated, semi-wild and wild *Citrus* species for long-term.



Several studies have amply shown that Citrus seeds are non-orthodox in seed storage behaviour, therefore, are sensitive to desiccation and freezing (Malik et al., 2003). However, successful protocols have been developed for the cryopreservation of seed, embryos and embryonic axes of several species in various laboratories of the World (Malik et al., 2012; Normah et al., 2015). In India at ICAR-National Bureau of Plant Genetic Resources, successful cryopreservation and cryobanking of most of the Indian species of Citrus has been undertaken during last 2 decades based on developed cryopreservation protocols. Experiences and learnings from these studies have been compiled in the form of Standard Operating Procedures (SoPs) for the collection and conservation of citrus germplasm. These SoPs would be extremely useful for the utilization of developed protocols on routine basis for management of Citrus germplasm by students and scientists who wish to start research work in these areas. These SoPs would also be useful for all the partners of recently launched DBT sponsored mega-project on "Collection, Conservation and Morpho-Phenological Characterization of Citrus Germplasm of North East Region" being implemented at HUB (AAU-Citrus Research Station, Tinsukia), Mirror sites (ICAR-National Bureau of Plant genetic Resources, New Delhi and Umiam) and various spoke centres in NE States of India. Citrus genetic resource of NE India would be collected, characterized and conserved under this project in the Field Gene banks and also cryogenebank at ICAR-NBPGR and AAU-CRS, Tinsukia. The unique and comprehensive study under this project would provide excellent opportunity to the investigators to collect and conserve the genetic diversity of Citrus for posterity and utilization of germplasm for citrus improvement.

These SoPs have been developed keeping in mind the following issues which may be encountered during the collection and conservation of Citrus germplasm.

- Collection of fruit germplasm needs meticulous planning for exploration and collection as type of germplasm to be collected, fruit maturity and habitat plays important role in bringing healthy material to laboratory.
- Fruits/seeds being a perishable material post-collection care and handling of germplasm in the field and laboratory is critical.
- Seeds being non-orthodox, precocious germination occurs in several cases.
- Large seed size and limited availability of seeds per fruit puts collectors in dilemma to collect or not to collect the available germplasm
- There is difficulty in getting desirable accession size to conserve the total spectrum of genetic variability.

Improper collection and handling of germplasm which results in low initial viability and poor seed health lead to wastage of all efforts and unfulfilled targets.

Factors contributing to improper collection and handling of materials are:

- Poor understanding about the morphology and physiology of fruit/seeds
- Collection of fruit/seeds at physiological maturity not suitable for conservation
- Inappropriate seed extraction and storage during collection or in the laboratory
- Use of inappropriate method for seed extraction and drying
- Infection of material by saprophytic fungi and bacteria causing low initial seed viability, poor storability and genetic deterioration

Further, Cryobanking of non-orthodox seed species is critical as understanding of seed characteristics, seed physiology and storage bahaviour plays important role in developing the protocol. In citrus seed morphology, physiology and sensitivity to desiccation and freezing remarkably varies on species-to-species basis or some times genotype to genotype. Therefore, suitability of cryopreservation technique and development of protocol with highest regeneration with normal plantlet/seedlings and repeatability of cryo-procedure is to be given desired attention. Similarly, establishment, maintenance and management of citrus Filed Gene Bank (FGB) also requires meticulous skills and procedures. Citrus being a very sensitive fruit crop there is a need to follow all the suggested protocols timely and correctly for keeping the plant healthy. SoPs developed and presented in this publication may address these issues and help the researchers in ease of doing collection and conservation of citrus germplasm.

2. Exploration and Collection

Materials required

a. Binoculars
b. Blotting Sheets
c. Camera
d. Cloth bags, rubber bands
e. Exploration kit bag
f. Field compass
g. First aid kit
h. Global Positioning System (GPS)
i. Hand gloves
j. Labeling tags
k. Magnifying lens
l. Markers
m. Measuring scale
n. Passport data book
o. Polythene bags
p. Secateurs
q. Sharp pocket knife
r. Water bottle

Information collection from farmers is crucial

2.1. Planning for Exploration

Exploration Team: Exploration and collection team should be a cohesive small group of 2-3 persons having good knowledge of citrus plant type, morphology and physiology. Having knowledge of taxonomy and habitat would be desirable for targeted and specific collections.

- The explorers should be well-versed with the nature, extent of diversity and timing of flowering-fruiting of species to be collected.
- Exploration should be undertaken when the fruits are physiologically mature and ready for harvest.
- Eco-geographic information needs to be gathered to finalize the itinerary of collecting mission.
- The information on areas to be explored for targeted species/ genotypes should be gathered from different sources.
- GIS Tools may be used for planning the exploration. This would help in identification of gaps in existing collection, to locate the specific geographical condition and

planning of collection trip by providing details of village maps, roads and other topographical details.

- Interaction and discussion with local people and progressive farmers may help in gathering relevant information about indigenous citrus diversity.
- Survey of local markets to have a look at the citrus fruits and other products being sold will provide useful information.
- If the germplasm is to be collected from protected and restricted areas, prior permission should be taken from the concerned authorities.

Interaction with progressive farmers

2.2. Collection of Germplasm: How to Collect? What to Collect?

2.2.1. Fruits/Seeds

1. Fruits/seeds from trees marked at the time of flowering from field gene bank, farmer's orchards, homestead gardens and natural habitats may be collected
2. Harvest the fruits directly from the trees
3. Fruits should be kept in muslin cloth bags to provide proper aeration and label the bag specifying collector number of sample
4. Record information in the passport data book at the site of the collection itself and gather the relevant information (Please see Passport data sheet for details)
5. Collect herbarium specimens and take photographs of plant type, leaf, flower and fruits

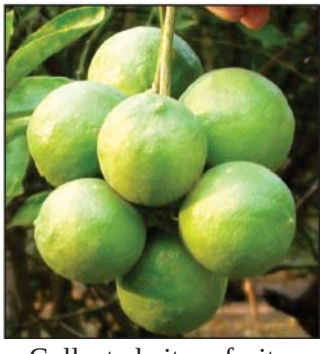
Note

- The collection site should be identified accurately using the map.
- An adequate number of fruits must be collected based on the number of seeds available per fruit.
- Fruits showing signs of infestation must not be harvested.
- The collection bags should have labels both inside as well as outside of the bag.
- It is important to press the herbarium specimens on the same day of collection.
- Take morphological data of collected samples on the same day of collection, if possible.
- The observations should be recorded based on descriptors, in case, it is not possible to record all descriptors important morphological traits may be recorded.
- Collected data and information should always be recorded on passport data book or additional notebook.

Collection from natural sites

Collection from Field genebank

Fruit harvesting



Collected citrus fruits

- During the exploration mission, the work done throughout the day may be reviewed and discussed on the same evening and planning for the next day may also be done based on information available from various sources.

Points to Remember

1. During exploration mission, keep your identity card and if desired a certificate from the Head of organization mentioning your official duty details.
2. A four-wheel drive vehicle (with all the safety equipment as per the topography of region to be explored) should preferably be used for the exploration mission.
3. While planning to explore in the forest areas, remote and difficult localities and tribal inhabiting areas, the explorer should take help from local government officials, NGOs and village authorities before starting the collection of germplasm.
4. Before collection, concerned farmers and local peoples should be taken in the confidence by introducing the team and explaining them the purpose of collection. The germplasm should not be collected without the consent of the concerned person/ farmer/ Biodiversity Management Committee (BMC).
5. Always be polite and respectful to the farmers and tribal people during the conversation and discussions.
6. Respect the social beliefs and customs of the local people of the target area and appreciate their efforts of supporting you.
7. Strictly restrict your conversation with the local people on the topic of collection and general agricultural practices etc.
8. Never make false promises with farmers in return of knowledge extraction and/ or germplasm from them.
9. Always carry proper and appropriate clothes and other wearables according to the weather and climatic conditions of the targeted area to be explored.
10. While going to the forest areas, do not forgot to carry mosquito repellents, anti-leech lotions, knee socks etc. to protect yourself.
11. An exploration mission should never be planned during important local festivals and elections in the particular target area.
12. Never eat unknown wild fruits since some of them may be poisonous or harmful to health. Avoid eating unhygienic food to remain healthy during exploration.


Citrus fruits for collection

Collection of citrus fruits

Collection of citrus fruits


Data entry in passport data book

NBPGR

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Pusa Campus, New Delhi-110 012

PASSPORT DATA FORM

ICAR

Collector's Name and Address:
Collaborating Institute: Name of Scientist(s) and Address:
Area Explored:
Duration of Exploration: From……. **To:**…….

Sr. No.	Collector No.	IC No.	Crop's common name	Botanical name	Cultivar/ Landrace name	Biological status	Type of material	Collection date	Collecting site /acquisition source	Frequency
1.										
2.										

Sr. No.	Collector No.	Sample type	Sampling method	Habitat	Site of collection				Latitude (N)	Longitude (E)	Altitude (m)	Ethnobotanical information/ Ethnic group	Remarks (Trait-specific characters)
					Village	Mandal/Taluk/ Tehsil	District	State					
1.													
2.													

For allotting IC number the completed sheets should be sent along with samples (2000/4000 seeds of self/cross pollinated crops) to: **The Head, Division of Plant Exploration and Germplasm Collection, ICAR- National Bureau of Plant Genetic Resources (old building), Pusa Campus, New Delhi-110 012.**
E mail address: NBPGR.exploration@icar.gov.in (please also send soft copy of passport data in MS Excel through e mail), Phone: 011-2584 8405 (O)
For taking IC number to the vegetatively propagated crops/species, **also furnish the certificate of conservation and maintaining in field gene bank/NAGs**

Useful tips

Collector number: denotes a unique/primary identity assigned by the collector at the time of collection, given in abbreviated form of collectors name followed by accession number (for example: KCB/KP/231 (expanded form is: collector number assigned in an exploration by KC Bhatt as team leader and K Pradheep as collaborator/associate; germplasm sample sequence/ serial number is 231)
Biological status: (***Wild***-All wild species that are related and part of the genepool from which genetic introgression into cultivated species is possible using conventional methods; ***Weedy***-Weedy form of cultivated species occurring in companionship (fields) of some other cultivated species; ***Landrace/Traditional cultivar/ Primitive cultivars/ Farmers variety***- All cultigens under cultivation in farmer's field with/ without specific names frequently associated with unique traits identified by farmers; ***Breeding line***-Semi-finished products or segregating material generated out of hybridization programme to meet specific breeding objectives; ***Elite line/Advanced/ Improved cultivar***- Selection from population, from coordinated trial (AVTII line), improved cultigens of common knowledge in commercial cultivation (extent, released by institution/organization/State) but not notified from the Central Sub-Committee on crop standards, Notification and Release of Varieties of Agricultural and Horticultural Crops and Parental lines of hybrids; ***Released cultivar/ Hybrid***-Varieties/hybrids notified and released by the Central Sub-Committee on crop standards, Notification and Release of Varieties of Agricultural and Horticultural Crops; ***Genetic stock/ Registered germplasm***- Trait and gene specific germplasm experimentally developed/identified through scientific interventions (e.g. sources of resistance, mutant, cytogenetic stock etc.) which is registered for unique trait(s) at ICAR-NBPGR; ***Others***-Doubtful or material with unknown biological status)
Type of material: Seed/fruit/inflorescence/root/underground parts/cuttings/live plants
Collecting site/acquisition source: Farmer's field/ threshing yard/ fallow/ farm store/ wild/ orchard/home garden/ market/ aquatic/Institute name (if others, give source name)
Frequency: Abundant/frequent/occasional/rare; **Sample type:** Population/pure line/individual; **Sampling method:** Bulk/random/non-random/individual plant
Habitat: Cultivated/disturbed/partially disturbed/rangeland/forest/aquatic habitat. Fill the **latitude and longitude** in decimal points (ex. 75.23) instead of degree and minutes (75° 23').
Note: Please do not merge, delete, change sequence and content of columns in table. However, append additional rows as per need (number of accessions) in above table. Submit separate datasheet for explorations conducted by different collectors or at varying period. For breeding line/genetic stock developed in Institute, location of farm/institute to be filled.

2.2.2 Scion collection

1. Select bud wood from the last years flush only
2. Cut the twigs of same thickness of the rootstock available at field genebank/ orchard
3. Cut /trim about 25-30 cm long sticks and remove extra leaves flush
4. Wet collected bud wood should be immediately rapped in moss grass/ blotting sheets and then keep in polythene bags
5. Store and transport the bud wood at 15-20° C temperature

Note

- The scion should be selected from a mother plant which is already in its reproductive phase of growth.
- Bud wood must be collected when cell sap is running, bark may be easily peeled away and buds are dormant.
- Twigs must be collected from the last grown flesh having mortality markes.
- Leaves should be cut of leaving petiole stub about 0.30 cm long to protect the bud from injury.
- Bud wood should be round, straight, smooth and of minimum pencil thickness with well-developed buds.
- Collected bud wood may be waxed to provide extra protection from water loss.

Points to Remember

1. The moist bud wood may be blotted with proper towel paper to remove extra moisture.
2. Shriveled dark colored or heavily molded bud wood should be immediately discarded.
3. After collection bud wood should be wrapped properly with polythene sheets to avoid moisture loss and tie the bundle with rubber band/ soft thread.

Selection of scion bud wood

Extra leaves remove from bud wood

Collected fresh bud wood with 4-5 buds

Wrapped with paper towel/blotting/ news paper

Waxing

Rolled collected twigs/bud wood

Packaging of bud wood scions

Twigs/ bud wood Handling in laboratory

2.3. Post-harvest Handling and Transport

Materials required

a. Cloth bags

b. Cardboard boxes

c. Record book

d. Sawdust/charcoal/peat moss

Packaging of citrus fruits

Procedure

Ensure freshness of fruits by storing them in an airy, shaded and cool place until transported to laboratory

↓

Transportation of fresh fruit germplasm

↙ ↘

Transport the fresh fruits immediately after collection to the laboratory in cloth bags/ placed in cardboard boxes

If transportation of whole fruit is not possible then pack the freshly extracted seeds in the juice of same fruit/ sawdust/ charcoal/ peatmoss and transport to the laboratory as early as possible

Note

- The fresh germplasm may be susceptible to very high or the very low temperatures. Moderate temperature should be maintained during transport and aerated cardboard boxes may be used.
- Whole fruits should preferably be transported to lab, incase of bulky fruits seeds dipped in the fruit juice-pulp may be carried or transported.

Collected citrus fruits

Packaging of collected fruits

3. Post Collection Handling in Laboratory and Characterization

Materials required

a. Descriptor

b. Measuring scale

c. Pencil, pen and marker

d. Record book

e. Refrigerator/ BOD

f. Refractometer

Handling of citrus fruits in laboratory

3.1. Laboratory Storage

Procedure

1. Confirm the labels from the list or passport data of collected germplasm
2. Wash the fruits with warm water and clean them using muslin cloth. In case fruits need to be stored for long time, surface sterlize them with any broad-spectrum fungicide and/or 70% alcohol
3. Citrus fruits/seeds should be stored (in bags with proper labels) in mild cold conditions (15-20°C) and humidity until processed for cryobanking

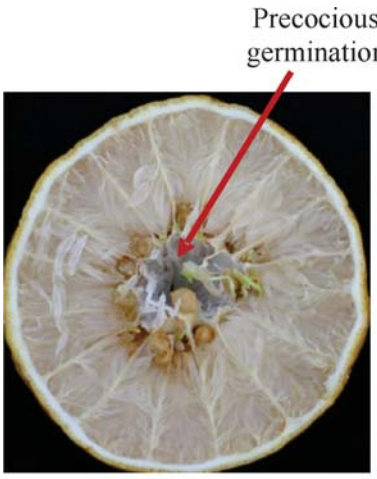
Note

- The extracted seeds should be processed at the earliest possible time.
- While cleaning the extracted seeds, handle them carefully and prevent any physical damage to them.
- If delay in seed processing is expected, then special care needs to be taken to maintain seed viability and to avoid precocious germination during storage.

- Since vivipary exists in citrus, the seeds cannot be left within the fruit for a long time that may lead to germination inside the fruit itself.

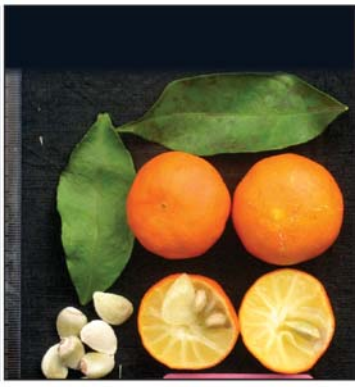
Points to Remember

1. During transportation, some fruits/seeds may get infected by insect-pest. Such seeds must be carefully segregated during processing and should not be stored as such.



Vivipary in ***Citrus*** species

Seeds of *C indica*



Leaf, Fruits and Seeds of *C. indica*

3.2. Characterization of Leaf, Fruits and Seeds

1. Record leaf, fruit and seed characters using Bioversity International or any other suitable Citrus descriptors
2. Morphological data in replicate of three to five leaf/ fruits/ seeds is to be recorded for collation and analysis
3. Data should be recorded using characterization descriptors and quantitative characters such as total soluble solids (TSS), acidity etc. should be measured using standard instruments and RHS Color Chart may be used for color depiction

Note

- It is better to record the characterization data at the earliest, so that least errors are occur in the data.
- The characterization data recorded on additional descriptors other then listed in standard descriptor being followed must be specified.
- The characterization data should not be altered for one's own convenience.

Characterization of *Citrus* species

Points to Remember

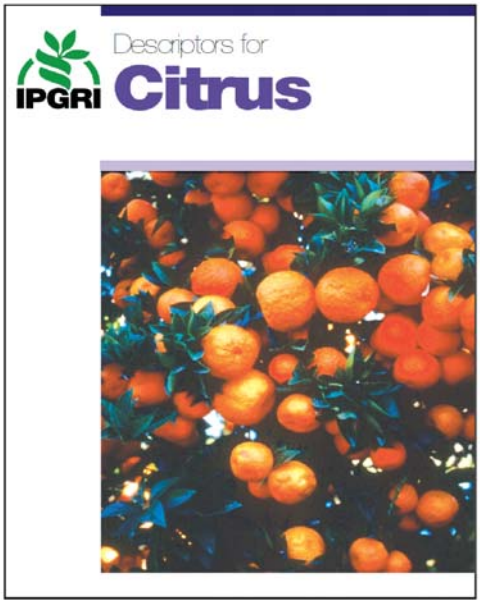
1. Correct identification of Species/ Sub-species/ Cultivar is very important in citrus.
2. Characterization data should only be compared within species or group of citrus.

Sample data recording

Sample Data Sheet

Characterization	Collector No. MD-190 Accession no. IC 417164			Collector No. MD-200 Accession no. 417166		
Fruiting season (7.4.1)	3	3	4	3	3	4
Fruit weight (g) (7.4.3)	25.41	18.86	21.42	11.17	11.02	12.31
Fruit diameter (mm) (7.4.4)	35.00	32.00	33.00	29.00	27.00	29.00
Fruit length (7.4.5)	34.00	30.00	30.00	26.00	25.00	26.00
Fruit shape (7.4.6)	1	1	1	5	5	5
Shape of fruit base (7.4.7)	3	3	3	3	3	3
Shape of fruit apex (7.4.8)	3	3	3	4	4	4
Fruit skin colour (7.4.9)	4	4	4	5	6	6
Width of epicarp equatorial area (mm) (7.4.11)	1	1	1	1	1	1
Fruit surface texture (7.4.12)	1	1	1	1	1	1
Adherence of albedo to pulp (7.4.13)	3	3	3	3	3	3
Nature of Oil glands (7.4.14)	1	1	1	1	1	1
Density of oil glands on fruit surface (7.4.15)	7	7	7	5	5	5
Oil gland size on fruit surface (7.4.16)	3	3	3	3	3	3
Fruit rind thickness (mm) (7.4.17)	2	2	2	1.5	1.5	1.5
Albedo colour (7.4.18)	3	3	3	3	3	3
No. of segments per fruit (7.5.1)	7	7	7	6	6	6
Pulp colour (7.6.1)	3	3	3	4	4	4
Pulp firmness	3	3	3	3	3	3

No. of seed/fruit (7.7.1)	3	2	2	3	2	2
Seed shape (7.7.3)	2	2	2	3	3	3
Seed surface (7.7.4)	12.4	12.6	12.5	11.9	11.4	11.4
Seed colour (7.7.5)	1	1	1	1	1	1
Colour of cotyledons (7.7.6)	2	2	2	2	2	2
10 Seed wt. (gm)	1.67	1.90	1.59	1.58	1.60	1.63
Chalazal spot colour (7.7.7)	5	5	5	5	5	5
Seed embryony (7.7.8)	2	2	2	2	2	2
Seed moisture (%)	15	16	15	15	16	16
Leaf division (7.2.2)	5	5	5	6	5	5
Intensity of green colour of leaf blade (7.2.3)	3	3	4	3	3	4
Leaf colour variegation (7.2.3.1)	2	2	2	2	2	2
Leaf lamina attachment (7.2.4)	4	4	4	4	4	4
Leaf lamina length (cm) (7.2.5)	7.2	7.3	7.3	7.2	7.3	7.3
Leaf lamina width (cm) (7.2.6)	3.6	3.5	3.5	3.6	3.5	3.5
Ratio leaf lamina l/w (7.2.7)	2	2	2	2	2	2
Leaf thickness (mm) (7.2.8)	30	30	30	30	30	30
Leaf lamina shape (7.2.9)	2	3	3	2	3	3
Leaf lamina margin (7.2.10)	3	3	2	3	3	2
Leaf apex (7.2.11)	4	4	4	4	4	4
Absence/presence of petiole wings (7.2.12)	1	1	1	1	1	1
Petiole wings width (7.2.13)	3.1	3.2	3.1	3.2	3.2	3.1
Petiole wing shape (7.2.14)	1	2	2	1	2	2



IPGRI Descriptor for Citrus

Refractometer for TSS

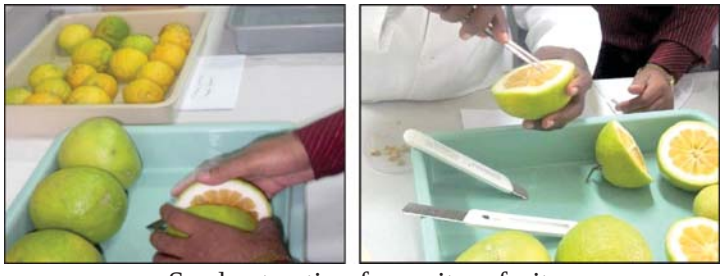
4. Basic Studies on Seed Characteristics

4.1. Seed/ Embryo Extraction and Excision of Embryonic Axes

4.1.1. Seed extraction

Materials required

a. Broad-spectrum fungicide
b. Charcoal
c. Forceps
d. Magnifying lens
e. Muslin cloth
f. Petri plates
g. Sharp knife
h. Saw dust
i. Trays
j. Warm water



Seed extraction from citrus fruits

Procedure

1. Give a cut in the middle of the fruit and tilt both halves, so as to avoid damage to the seeds and extract the seeds carefully
2. Wash the seeds with warm water and use muslin cloth to clean and remove any fruit part to avoid infection during storage
3. For temporary storage, seeds must be kept in sawdust, charcoal or in vials after treating with broad-spectrum fungicides at temperatures between 15 to 20°C till used for further experiments

Note

- The seeds which are to be used for moisture content determination should not be washed so that the actual moisture content is not affected.
- Once the seeds are extracted, try to use them for further procedures immediately or at maximum, within 1-2 days.

Extraction of seeds from citrus fruit

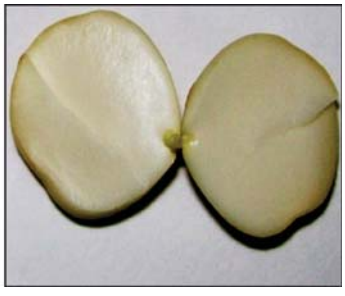
Extracted seeds

4.1.2. Extraction of Embryos

Materials required

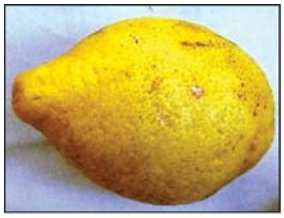
a. Blotting sheets
b. Magnifying lens
c. Sharp knife
d. Sterile petri plates
e. Sterile surgical blade
f. Warm water

Embryos of *Citrus*



Embryo showing cotyledons

1. Extract seeds from fruits as described earlier
2. Wash the seeds to remove any pulp adhered to the seeds
3. Air dry by spreading the seeds on blotting sheets to remove extra water
4. Delicately remove the seed coat and extract the embryo



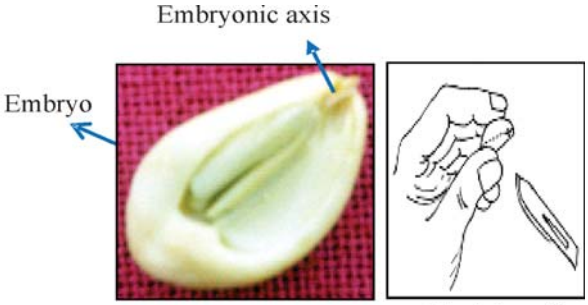
Note

- If the seeds are hard, soak them in water for 20 min so that it becomes easier to extract the embryos.
- The embryo should be extracted carefully without damaging the embryonic axis.

4.1.3. Excision of embryonic axes

Materials required

a. Fungicide
b. Hand gloves
c. Laminar air flow cabinet
d. Magnifying lens
e. Sharp knife
f. Sterilant- Sodium hypochlorite (2%)
g. Sterile distilled water
h. Sterile surgical tools
i. Sterile petri plates



Excision of embryonic axis

Procedure

1. Extract sufficient number of seeds from fruits
2. Seeds should be treated with 0.2% Bavistin for 5-10 min
3. Rinse 3-4 times with sterile distilled water
4. Surface sterilize the seeds with Sodium hypochlorite (2%) for 10 min
5. Rinse the seeds four times with sterile distilled water
6. Delicately remove the seed coat to separate the cotyledons and split one of the affixed connections with embryonic axis
7. Separate out the embryonic axis by incising its other affixed connection

Note

- Morphology of citrus seeds should be well known for proper excision of embryonic axes.
- The whole procedure should be performed under aseptic conditions in laminar air flow cabinet.
- Excise the embryonic axes carefully without damaging the radical (root) or plumule (shoot).
- Time gap in excision of embryonic axes should be minimum to avoid unequal desiccation of excised embryonic axes.
- In case embryonic axis is deep seated, study the basic histology by taking a hand cut section and viewing it under the microscope.

Points to Remember

1. Wear gloves while handling fungicides and other surface sterilants.
2. Do practice of embryonic axes excision before undertaking experiment.

4.2. Moisture Content Determination

Materials required

a. Hot air oven

b. Moisture bottles

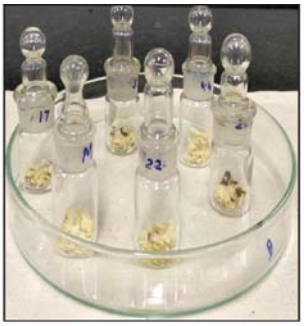
c. Mortar and pestle

d. Glass petri dishes

e. Record book

f. Weighing balance

Weighing balance



Moisture bottles containing pieces of citrus seeds

Procedure

1. Moisture content of seeds and embryos should be measured using hot air oven method (ISTA, 2015)

2. Determine the fresh weight of small pieces of seeds in three replicates after placing them in a moisture bottle, then dry in a hot air oven set at 103±2°C for 17 h, cool in desiccator and reweigh

3. Loss of weight equivalent to the amount of water is measured

4. Moisture content should be calculated using the following equation:

$$\% \text{ Moisture content on fresh weight basis} = \frac{\text{Fresh weight before drying-Fresh weight after drying x 100}}{\text{Fresh weight of sample}}$$

Note

- The seeds/ embryos can be desiccated in desiccator/ vacuum desiccator by placing them in muslin cloth, petri plates, on filter paper or on top of the aluminum foil.
- The muslin cloth containing seeds or embryos should be loosely tied for proper aeration and equal desiccation
- Initial moisture content (IMC) is obtained after this procedure.

4.3. Viability Testing

4.3.1. Viability testing of seed, embryos and embryonic axes

There are different methods for testing viability of seed, embryos and embryonic axes

4.3.1.1. TTC Staining

Materials required

a. Camera

b. Chemicals- 2,3,5-Triphenyl tetrazolium chloride (TTC)- 0.2-0.6% (w/v), water, phosphate buffer (0.05 M Na_2HPO_4–KH_2PO_4, pH 7.4)

c. Glass petri plate

c. Incubator

d. Stereoscopic Microscope

e. Surgical blade

Red stained Citrus seeds with embryonic axes showing good viability

Procedure

1. Export seeds, embryos or excise embryonic axes
2. Dip seeds/ embryos in TTC solution [0.2-0.6% (w/v) TTC prepared in either water / phosphate buffer (0.05 M Na_2HPO_4–KH_2PO_4, pH 7.4)] in amber bottles
3. Incubate in dark at 30°C overnight
4. Rinse the explants using distilled water and observe under a stereoscopic
5. TTC staining (red color due to formation of Formazan in living tissue) indicates viability
6. Take the data and photographs of the seeds/embryos for record

4.3.1.2. Petri plate germination

Materials required

a. Distilled water

b. Filter paper

c. Forceps

d. Fungicide

e. Petri plates

f. Seed germinator or BOD

Citrus indica *Citrus aurantifolia*

Procedure

1. Treat the seeds and embryos with 0.2% Bavistin for 5-10 min
2. Place the seeds and embryos between two sheets of moistened filter paper in plastic Petri plates
3. Incubate at 27±2°C with 16/8 h light/dark photoperiod
4. Regularly (twice in a week) moisten the filter papers using distilled water and observe/ record the condition of the seeds and embryos
5. Emergence of shoot and/or root indicates germination

Note

- Do not spray too much water on the filter papers as it may lead to decay and contamination of the seeds and embryos.
- Remove the contaminated seeds and embryos, if any, from the petri plate.
- Record the germination data at regular intervals.

4.3.1.3. Paper towel germination in case of large seeds

Materials required

a. Butter paper

b. Distilled water

c. Forceps

d. Fungicide- 0.2% Bavistin

e. Marker

f. Paper towel

g. Seed germinator or BOD

Citrus ichangensis seed germination

Procedure

1. Soak the towel papers properly in distilled water
2. Treat the seeds/embryos with 0.2% Bavistin for 5-10 min
3. Take a butter paper and fold it in half. Label it with complete details
4. Place two wet towel papers over the butter paper
5. Place the treated seeds/embryos on the towel paper at equal distance using a forcep. Cover them with one wet towel paper sheet
6. Roll the butter paper with towel papers carefully in such a way that the labelling is visible in front. Place the rolled towel papers in the seed germinator
7. Regularly (once in a week) moisten the towel papers using distilled water, observe the condition of the seeds/embryos and record data
8. Evaluate the germination for normal, abnormal and dead seeds as per ISTA guidelines

Note

- Do not spray too much water on the towel paper as it may lead to contamination of the seeds and embryos.
- Remove the contaminated seeds and embryos, if any, from the towel paper.
- Take observations and data at regular intervals.

4.3.1.4. *In vitro* culture

For testing the viability of embryonic axes (EA), *in vitro* culture in suitable media is undertaken.

Materials required

a. Culture media- Murashige and Skoog (MS) macro and micro-nutrients, vitamins, iron, 1g L^{-1} activated charcoal and 0.17 g L^{-1} NaH_2PO_4 supplemented with 1 mgL^{-1} each of 6-benzylaminopurine (BAP) and á-naphthalene acetic acid (NAA), as defined by Chin et al. (1988).

b. Fungicide- 0.2% Bavistin

c. Hand gloves

d. Laminar air flow cabinet

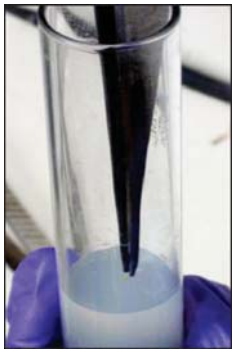
e. Sterilant-Sodium hypochlorite (2%)

f. Sterile Surgical tools

g. Sterile Petri plates

h. Sterile glassware

i. Sterile Test tubes



In-vitro culture of embryonic axis

Procedure

1. Surface sterilize, seeds and excise embryonic axes (Refer Section 4.1.3). Culture the embryonic axes on culture media in aseptic conditions in laminar air flow cabinet
2. Maintain the cultures in dark for 5-7 days at 25±2°C and shift to 16 h photoperiod under a light intensity of 35 µE m-2 s-1
3. Assess the growth after 3 weeks of culture and at regular time interval

Note

- The whole procedure should be performed under aseptic conditions.
- All the glasswares and surgical tools used for working under laminar flow should be sterile.
- Switch on the UV light in the laminar air flow 15-20 mins before starting the work.
- The air flow of the laminar cabinet should be left switched on until the work is finished.
- Clean the work station with sterilant or 70% ethanol before starting the procedure.
- Do not completely push the EA in to culture media.
- The mouth of the test tubes should be covered with cap and sealed by cling film/ parafilm before taking them out of the work station.

Points to Remember

1. Cover the laminar air flow cabinet with a black cloth while UV light is switched on or take any other suitable step to avoid explore to UV light.
2. Do not touch the mouth of the test tube it may lead to contamination.
3. Be careful while working with alcohol and flame in the cabinet.

4.4. Desiccation

4.4.1. Desiccation of seeds and embryos

Materials required

a. Desiccator/vacuum desiccator filled with charged silica gel

b. Muslin cloth/aluminium foil/petri plate

c. Timer

Seed desiccation over charged silica gel

Procedure

1. Desiccate whole seeds and embryos in the desiccator/vacuum desiccator filled with charged silica gel
2. Standardize the duration of desiccation depending upon the initial and desired moisture levels
3. After each desiccation interval, determine the moisture content of seeds and embryos to obtain the **Critical moisture content.** Ideally desiccate the seeds and embryos to 10-14% moisture content
4. Determine the viability of seeds /embryos by petri plate germination/paper towel method/*in vitro* culture to obtain the maximum viability levels (minimum viability levels should be >50% of fresh control)
5. Store the seeds/embryos in the cryovials and maintain them in the desiccator till the next step is performed

Note

- Make sure that the silica gel is charged properly well in advance.
- The muslin cloth containing seeds or embryos should be loosely tied for proper aeration and equal desiccation.
- The seeds and embryos cannot undergo freezing until they are capable of tolerating desiccation down to 10-14% moisture content.

Points to Remember

1. The explants should never be placed in uncharged silica gel.
2. The time duration of desiccation should be followed attentively.
3. All the seed/embryos being desiccated should be same lot having equal moisture content levels.

4.4.2. Desiccation of embryonic axes

Materials required

a. Ethanol 70%

b. Laminar air flow cabinet

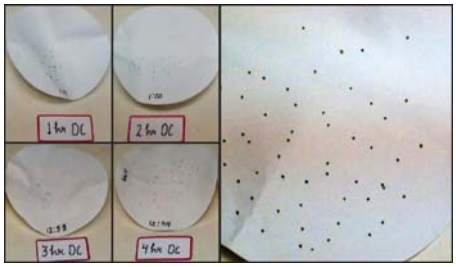
c. Sterile distilled water

d. Sterile filter papers

e. Sterile glassware and surgical instruments

f. Sterile petri plates

g. Timer



Desiccation of embryonic axes of *Citrus megaloxycarpa*

Procedure

1. After excision, keep the embryonic axes in batches of 20-25 in the laminar air flow cabinet immediately
2. Desiccate the axes for 1 to 6 h depending upon the size of axes, the initial and desired moisture content levels
3. After each desiccation intervals, moisture content and viability of embryonic axes need to be determined to obtain the **Critical moisture content (CMC)** and above 50% **viability levels**
4. Viability of desiccated axes should be determined by in vitro culture in the prescribed culture media

Note

- The embryonic axes must be excised under aseptic conditions.
- All the glassware and surgical tools used while working under laminar flow should be sterile.
- Switch on the UV light in the laminar air flow 15-20 mins before starting the work.
- The air flow of the laminar cabinet should be left switched on until the work is finished.

- Clean the work station with sterilant/ 70% ethanol before starting the procedure.
- The time duration of desiccation should be followed attentively.

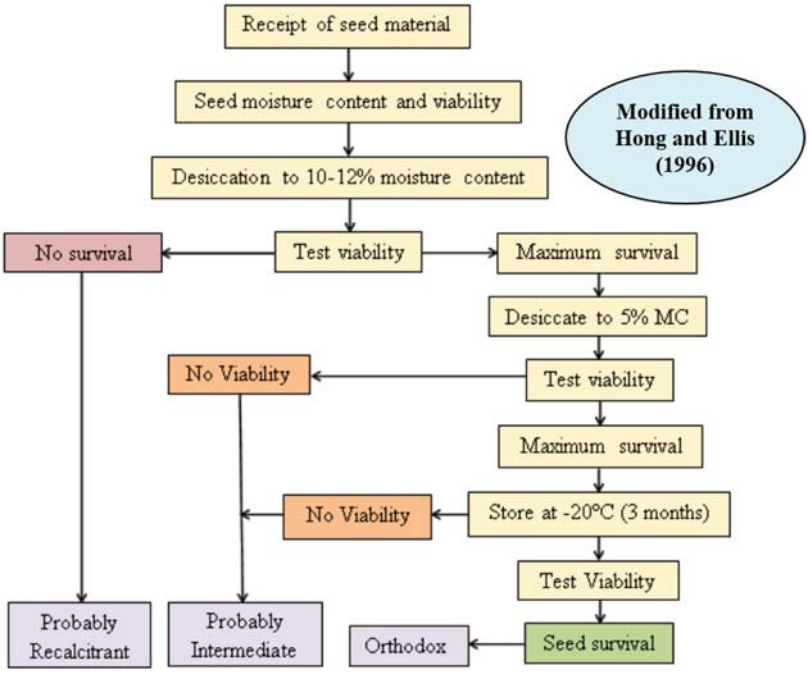
Points to Remember

1. Cover the laminar air flow cabinet with a black cloth while UV light is switched on.
2. Time duration between excision of EA should be minimum

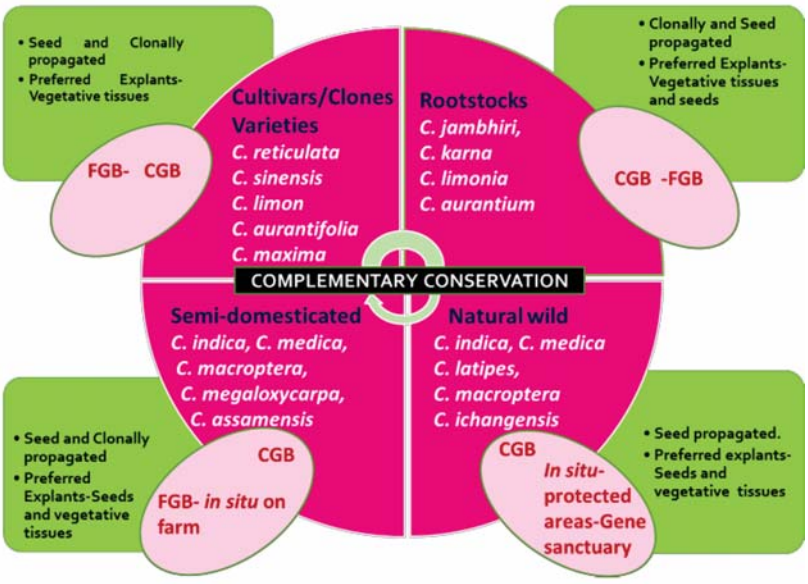
4.5. Determination of Seed Storage Behavior

- In order to determine the seed storage behavior, desiccate the explants at various intervals and obtain the Critical Moisture Content **(CMC)** and viability above 50 % **(P_{50})**.
- Depending on the CMC and P50, the seeds can be classified as orthodox, intermediate or recalcitrant (**Ellis *et al*., 1990)**
- There are two practical aspects to contrasting patterns of seed storage behaviour: first, the effect of desiccation on seed viability; second, the response of seed longevity (period of seed survival) to the storage environmental condition.

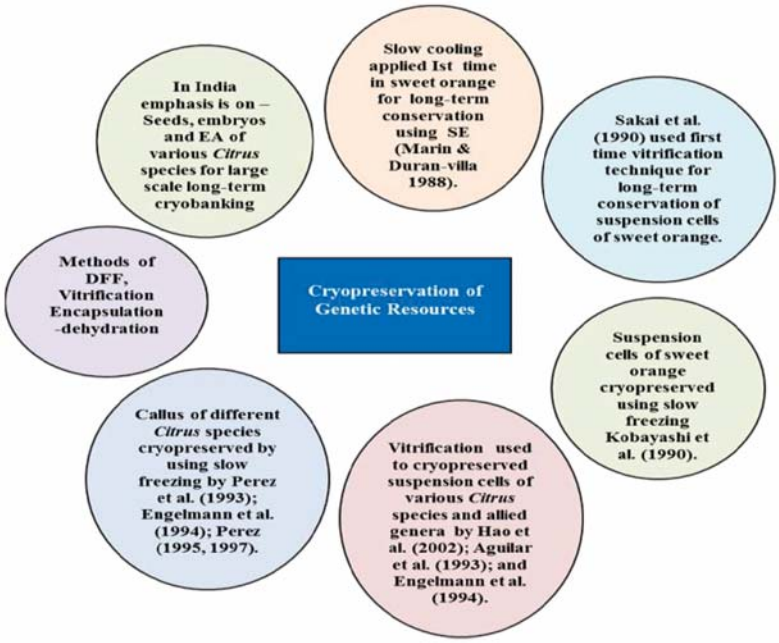
Protocol to Determine Seed Storage Behaviour



Candidates for Conservation for Citrus GR



Cryopreservation of various citrus explants



5. Cryopreservation of Seed and Embryos

5.1. Air Desiccation Followed by Fast Freezing

Materials required

a. Dewar flasks containing liquid nitrogen

b. Laminar air flow cabinet

c. Murashige and Skoog (MS) culture medium in glass test tubes

d. Sterile cryovials and cryo markers

e. Sterile filter paper discs

f. Sterile glass petri dishes and 100 ml flasks

g. Sterile surgical instruments

h. Timer

Procedure

1. Use the extracted seeds/embryos for viability testing-apply already standardized method
2. Desiccate seeds or embryos in the desiccator/vacuum desiccator filled with charged silica gel
3. Standardize the duration of desiccation depending upon the initial and desired moisture levels. It may vary from 4 h to 48 h to desiccate embryos to 10-14% moisture content
4. After each desired moisture content level, seed/embryos are either used immediately for viability testing and freezing or stored in the tightly screwed cryovials in desiccator for later use
5. Test the viability of seeds after each desiccation period using standardized method to ascertain the desiccation sensitivity and optimal MC for freezing
6. Desiccated embryos (about 10-15) with optimum moisture levels kept in 2/5 ml cryovials should be plunged rapidly in liquid nitrogen (LN)

contd.....

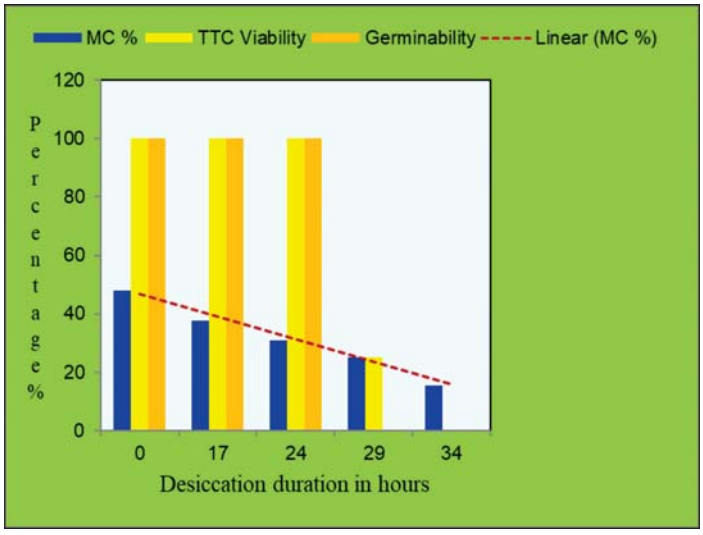
7. Cryostore in LN for minimum 48 h
8. Thaw the cryovials by fast plunging in a water bath maintained at 38°C for 5 min
9. Place seeds/embryos for petriplate germination immediately after thawing using standardized method
10. Record seeds/embryos germination data as described earlier

Note

- The time duration of desiccation should be monitored carefully to ascertain optimal moisture content suitable for freezing and to study desiccation tolerance.
- Desiccated seed/embryos should be carefully handled during protocol development to understand desiccation and freezing tolerance.
- During freezing and thawing fast plunging in LN and warm water, respectively, should be ensured for the optimal results.

Points to Remember

1. Wear gloves and face screen while handling fungicides and LN.
2. Avoid direct contact with LN, while plunging the cryovials in liquid nitrogen.



Representative diagram depicting effect of desiccation on MC, viability and germinability of Seeds/ embryo

5.2. Regeneration of Seeds and Embryos

Materials required

a. Culture media- Murashige and Skoog (MS) macro and micro-nutrients, vitamins, iron, 1g L-1 activated charcoal and 0.17 g L-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)

b. Distilled water

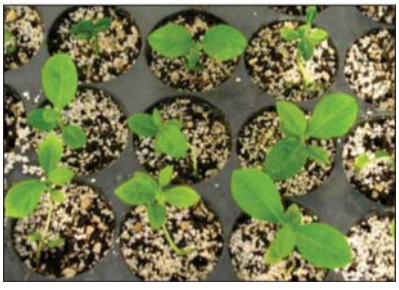
c. Fungicide- 0.2% Bavistin

d. Laminar air flow cabinet

e. Petri plates

f. Paper towel

Regeneration of healthy seedlings



Procedure

1. The regeneration can be evaluated by *in vitro* method for embryonic axes (Refer section 4.3.1.4) and by ISTA methods for whole seeds/ embryos (Refer section 4.3.1.2 & 4.3.1.3)

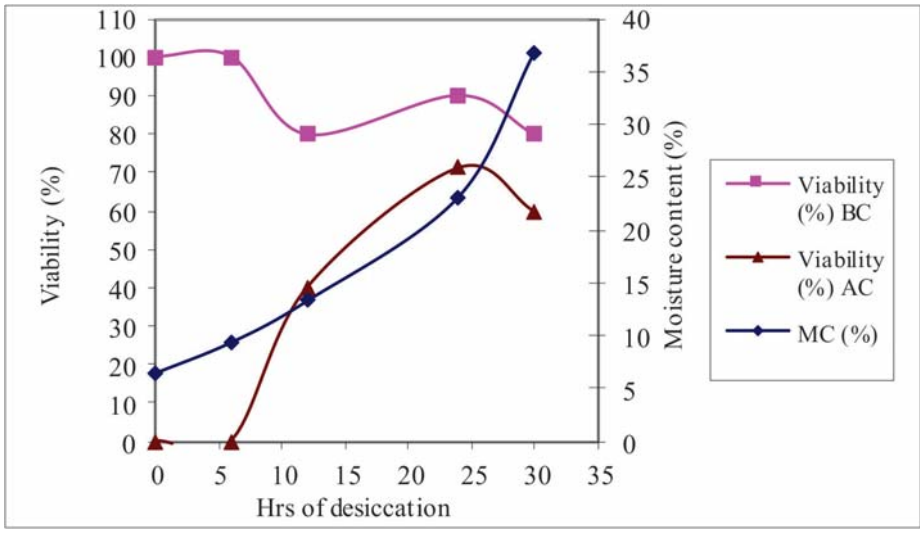
2. Observe and record the growth of the seedlings on a regular basis

Petri plates seed/embryo germination

In vitro Regeneration of *Citrus macropetra*

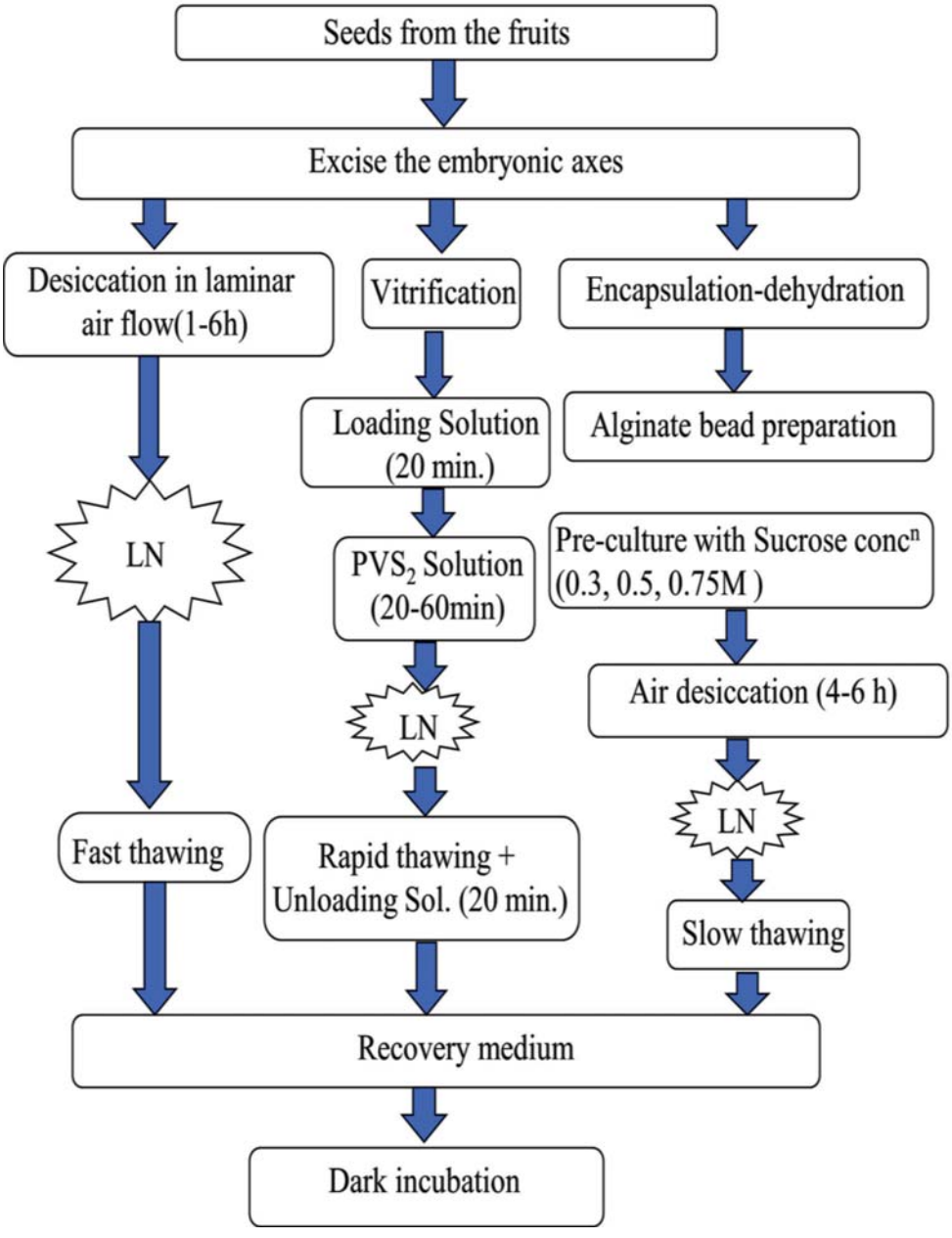
Note

- Petri plates/*in vitro* cultures containing frozen seeds/embryos should be kept in dark for first 3-4 days and later shifted to normal light for better regeneration.
- Regeneration methods including media composition, culture conditions etc. may be standardized to get the optimum results.
- Healthy seedling or plantlet without any callus would be treated as normal regeneration ensuring successful development of protocol.
- *In vitro* culture should be performed under aseptic conditions in a laminar air flow cabinet.
- The seedlings which are morphologically abnormal must be noted as it could be a sign of stress of desiccation and/or freezing.



Representative diagram showing effect of desiccation on MC, viability before cryo and viability after cryo in seed/embryo

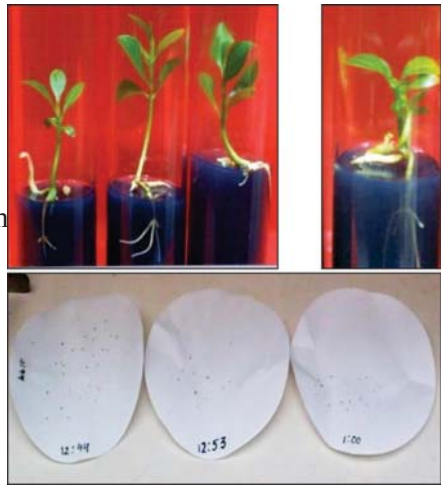
6. Cryopreservation of Embryonic Axes



6.1. Air Desiccation-Freezing of Embryonic Axes

Materials required

a. Dewar flasks containing liquid nitrogen
b. Ethanol 70%
c. Laminar air flow cabinet
d. Murashige and Skoog (MS) culture medium
e. Sterile cryovials and cryo-markers
f. Sterile filter paper discs
g. Sterile glass Petri dishes and 100 ml flasks
h. Sterile surgical instruments
i. Timer

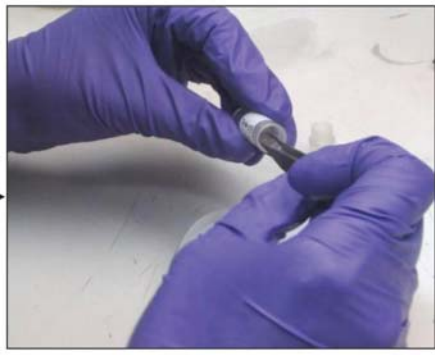


Air-desiccation of EA

Procedure

1. Excise the embryonic axis from embryos (Refer section 4.1.3)
2. Use 15-20 axes for moisture content determination using low constant temperature oven method
3. Spread rest of the axes on sterile filter paper discs in the air current of a laminar flow cabinet for various periods (1-6 hr) to bring the moisture to critical moisture content levels (CMC)
4. Measure the MC and viability after each (one hr) interval to ascertain the MC at which viability is above 50% of fresh control. This would also provide the desiccation sensitivity levels of EA
5. Place about 10-15 desiccated axes (at each hr) in sterile 1-2 ml cryovial, load these on canes and plunge rapidly in LN
6. Cryostore the vials in LN upto minimum 48 hours
7. Thaw the cryovials in a water bath kept at +38°C for 5 min by fast plunging
8. Culture the fresh and treated EA in recovery medium
9. Incubate the cryopreserved embryonic axes in dark for one week before exposing to full light

Air desiccation of citrus EA



Transfer of EA in cryovials

Immersion of cryovials in LN

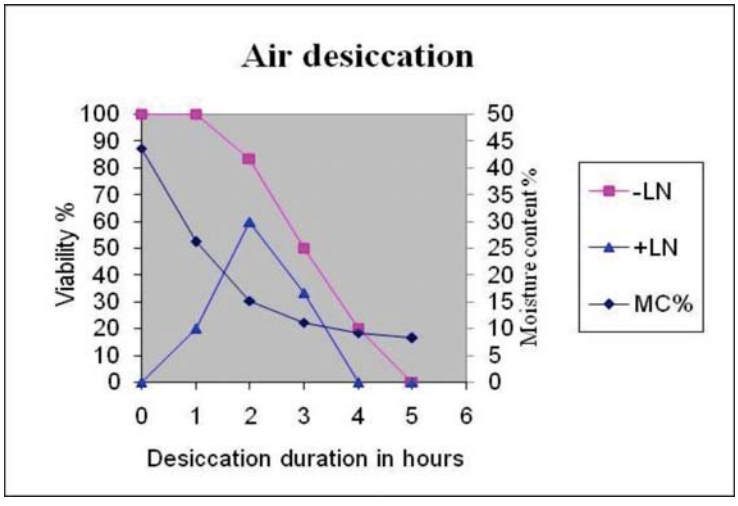
Note

- Switch on the UV light in the laminar air flow, 15-20 mins before starting the work.
- The work station should be cleaned using 70% ethanol before starting with the procedure.
- All the glasswares and tools used while working under laminar flow should be sterile.
- The air flow of the laminar air flow cabinet should be left switched on until the work is finished.
- The embryonic axes must be excised under aseptic conditions.
- Excise the embryonic axes carefully without damaging the root or shoot apex.

- The time duration of desiccation should be followed attentively.
- Keep the water bath ready at desired temperature before bring out the cryovials from LN for thawing.

Points to Remember

1. Cover the laminar air flow cabinet with a black cloth while UV light is switched on.
2. Avoid direct contact with LN, while plunging and getting the vials out from liquid nitrogen.



Representative diagram indicating effect on duration of desiccation MC and viability before and after cryo in EA

6.2. Vitrification

Materials required

a. Dewar flasks containing LN
b. Laminar air flow cabinet
c. Lab freezer
d. Sterile cryovials and cryomarkers
e. Sterile filter paper discs
f. Sterile glass petridishes and 100 ml flasks
g. Surgical instruments like scalpel blade holder, scalpel blades, forceps, needles, etc.

Regrowth of Embryonic axis

Solutions and Medium

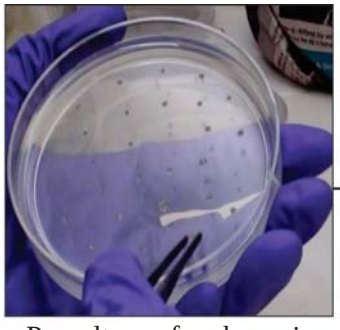
a. Preculture medium: basal MS medium supplemented with 0.3 M sucrose, 2M glycerol and 0.7% agar

b. Loading solution: 0.4M sucrose, 2M glycerol in liquid basal MS medium

c. PVS2 solution: 30% (w/v) glycerol, 15% (w/v) ethylene glycol, 15% (w/v) Dimethyl sulphoxide and 0.4 M sucrose in liquid basal MS medium

d. Unloading solution: 1.2M sucrose in liquid basal MS medium

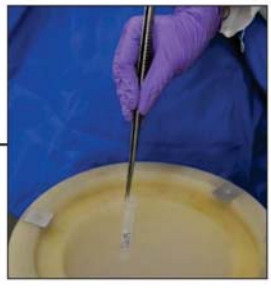
e. Culture medium: MS medium macro- and micro-nutrients, vitamins, iron, supplemented with 1g l^{-1} activated charcoal, 0.17g l^{-1} NaH_2PO_4 and 1mg l^{-1} each of BAP and NAA after Chin *et al.*, (1988)



Preculture of embryonic axes

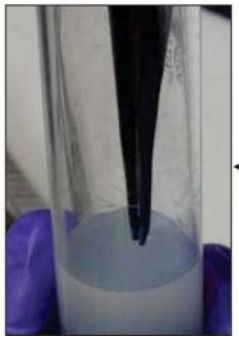
Transfer the embryonic axes in loading solution

Transfer of explant for PVS2 treatment at 0°



Plunge the cryovials in LN

Transfer the axes in unloading solution



Culture of explant

Procedure

1. Surface sterilize the seeds and aseptically excise embryonic axes (Refer section 4.1.3)
2. Preculture the axes on basal MS medium supplemented with 0.3 M sucrose and 2.0 M glycerol for 16 to 24 h and maintain in culture room conditions
3. Transfer 15-25 axes to sterile cryovials and treat them with loading solution for 20 min at 25°C
4. Replace loading solution with Plant Vitrification Solution-2 (PVS2) and incubate for 30 min at 25°C
5. Plunge tightly closed cryovials containing axes in PVS2 solution rapidly in liquid nitrogen (LN). Cryostore in LN for 48 h or more as per designed schedule
6. Thaw the cryovials in a water bath at 38°C±1°C by fast plunging for 5 min with vigorous shaking
7. Immediately replace the PVS2 solution with unloading solution. Leave the vials at 25°C for 20 min
8. Drain the solution and place the axes on sterile filter papers
9. Culture the axes in standardized culture medium and incubate in dark for one week before exposing to full light

Note

- Switch on the UV light in the laminar air flow, 15-20 mins before starting the work.
- The work station should be cleaned using sterilant/ 70% ethanol before starting the procedure.
- All the glasswares and tools used while working under laminar flow should be sterile.
- The air flow of the laminar cabinet should be left switched on until the work is finished.

- Treatment duration of pre-culture, loading solution and PVS-2 may be adjusted to get maximum re-growth.
- Concentration of pre-culture media can also be adjusted depending upon optimal results.

Points to Remember

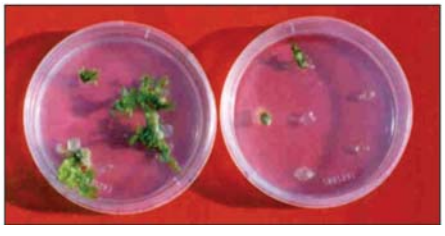
1. Wear gloves while handling PVS2 in order to avoid any contact.
2. Avoid direct contact with LN, while plunging the cryovials in liquid nitrogen and thawing.

6. 3. Encapsulation-Dehydration

Materials required

a. Autoclaved flask and beaker
b. Binocular dissecting microscope
c. Dewar flask and Liquid nitrogen.
d. Laminar air flow cabinet
e. Lab freezer
f. Petri dish lined with a filter paper
g. Sterile pipette tips to make beads
h. Sterile beakers
i. Sterile cryovials
j. Sterile forceps
k. Sterile flasks
l. Sterile petri plates 90 mm and 60 mm
m. Sterile scalpel with surgical blades
n. Sterile strainers

Encapsulated beads containing embryonic axes



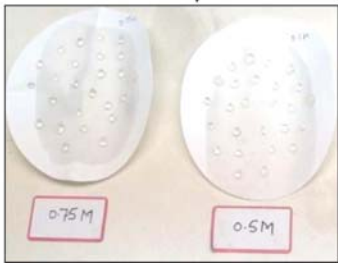
Solutions and Medium

- Sodium alginate solution (3%): MS medium with 0.5 M sucrose, 3% sodium alginate, pH 5.8
- Calcium chloride solution (100 mM): Liquid MS medium without halides, 100 mM calcium chloride, pH 5.8
- 0.75 M sucrose: Liquid MS medium containing 0.75 M sucrose, pH 5.8
- 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)

Explant in alginate solution

Single explant dispensed with a pipette

100 mM calcium chloride solution



Dehydration of beads in laminar flow cabinet

Immersion of cryovial in LN

In vitro culture of beads in recovery medium

Procedure

1. Surface sterilize the seeds and aseptically excise embryonic axes (Refer section 4.1.3)
2. Suspend the embryonic axis (EA) in calcium-free liquid MS medium containing 3% (w/v) sodium alginate
3. Dispense drops of sodium alginate solution containing one EA per drop with a pipette into liquid MS medium containing 100mM calcium chloride with
4. Solidify the beads for 15 min at 25°C with occasional stirring
5. Pre-culture the beads in liquid MS medium containing 0.75 M sucrose on a rotary temperature for 5-6 h in a laminar air flow cabinet
6. Remove beads from liquid medium and dehydrate in sterile petri plates at room temperature for 5-6 h in a laminar air flow cabinet
7. Determine the moisture content of the beads
8. Put the desiccated beads in 1.0 ml cryovials and plunge into liquid nitrogen
9. Thaw the vials slowly at room temperature for 20 min, culture the beads and incubate in dark for 7 days before exposing to light
10. Observe and record the data of survival and regrowth of embryonic axes

Note

- Switch on the UV light in the laminar air flow, 15-20 mins before starting the work.
- The work station should be cleaned using sterilant/ 70% ethanol before starting the procedure.
- All the glasswares and surgical tools used while working under laminar flow should be steriled.
- The air flow of the laminar air flow cabinet should be left switched on until the work is finished.
- Sodium alginate should be added in small quantities with constant stirring and mild heating to dissolve it completely.
- Duration of pre-culture and dehydration of beads may be adjusted to get maximum re-growth.
- Sucrose concentration in the pre-culture media may be adjusted to get optimal re-growth percentage.

Point to Remember

1. All the media/ solutions should be autoclaved and stored in refrigerator till further use.

2. Continuous stirring should be done while dropping embryonic axis containing sodium alginate solution in calcium chloride to avoid clumping of beads

7. Cryobanking

Packaging materials

7.1. Processing and Packaging

Materials required

a. Cryovials (1ml, 2ml, 5ml and 50 ml)

b. Refrigerator

Procedure

1. Seeds/embryos/EA at desired moisture content
2. For seed health test, critically observe the germplasm through visual examination and X-Ray radiography to obtain pest-free healthy seed
3. The explants should be finally packed in airtight containers before cryostorage
4. For packaging of seeds/embryos, polypropylene screwcap cryovials with inner thread should be used
5. Size of the cryovial should depend upon the size as well as the quantity of the explant to be stored
6. Seeds, excised embryo and embryonic axes should be stored in sufficient numbers in 1 ml/2ml cryovials

Note

- Damaged or infected seeds/embryos should not be packaged in cryovials.
- Cryovials should not be fully filled with explants, keep some empty place at the top.
- Do not pack entire sample in one cryovial. Replicates the sample in to small sizes, this would help in thawing only a part of sample when retrieved from cryotank. As ones thawed sample cannot be cryostored again.

- Part of sample should be treated as 'base collection' and should not be distributed or exchanged.

Points to Remember

1. Seeds/explants must be stored in air tight vials/containers in refrigerator till cryostorage.
2. Preferably, use cryovials with inner thread for cryobanking.

Packaging of seeds/embryo/embryonic axes in cryovials

7.2. Labeling and Cryobanking

Materials required

a. Cold resistant gloves
b. Closed circuit camera
c. Cryo Canisters
d. Cryocanes
e. Cryomarker
f. Cryolabels
g. Dry shipper/shipping container
h. LN tanks
i. Microprocessor liquid level controls
j. Safety glasses

Inventory systems

Procedure

1. After packaging, seeds/embryos/embryonic axes should be properly labeled in the space provided on cryovials
2. The label should consist of complete details *i.e.* crop name, collector no., IC/EC no., sheet no., storage location and date of storage on each cryovial
3. The cryovials should be clamped on canes. Then, place the canes in an cryo canister
4. Labeling should also be done properly on canes as well as on the cryo canisters
5. Store the canisters in cryotank in the vapour phase of LN at appropriate position

Note

- Storage location and IC/EC number of the sample is highly critical information. Be extra careful while labeling and writing these on cryovials. If possible use Bar code or QR code for labelling.
- Regular monitoring of cryotank temperature and LN level is must, daily recording of these two parameters should be undertaken.
- LN should be regularly replenished and ensured supply of LN should be ascertained for smooth functioning of cryobank.

Points to remember

1. Wear cryoprotection shield and gloves during handling liquid nitrogen.
2. Avoid direct contact with LN, while plunging the cryovials in liquid nitrogen.
3. LN must be used in a well-ventilated room to avoid the risk of suffocation.
4. Oxygen monitoring equipment should be installed where large capacity cryotanks are maintained in small room.
5. Closed circuit camera should be installed in the cryobank if possible.

Summary of Activities for Cryobanking

1. Handling and laboratory storage of germplasm in form of fruits, seeds, vegetative parts, etc.
2. Extraction and excision of explants
3. Estimation of moisture content
4. Standardization of germination procedure and viability measurement
5. Standardization of desiccation protocol
6. Standardization of freezing protocol
7. Storage of explants in cryotanks
8. Maintenance of cryostored germplasm & database
9. Retesting of cryostored materials after stipulated periods

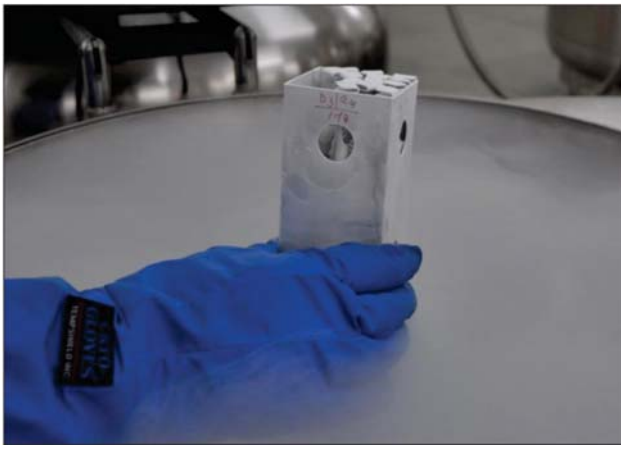
1. Filling of explant in cryovials

2. Labeling in cryovials

3. Cryovials plugged on canes

4. Canes placed in aluminum canisters

5. Canister shifted to LN tank



6. Properly labeled canister containing canes with cryovials in cryotank

7. Cryotanks

7.3. Database Entry and Management

Materials required

a. Database record book

b. Desktop/Computer

c. Passport databook

d. Suitably developed Database

Database entry at PGR portal

Procedure

1. Cryogenebank database should be user friendly and contain maximum possible information regarding the stored sample.
2. All the essential details like, collectors' no., IC/EC no., local name and botanical identity of samples, type of materials, location of storage and date of storage should be recorded
3. Original passport data of stored accessions should be linked to the cryogene bank database.
4. Botanical names and their spellings should be periodically checked for changes/updates
5. Written records of the main passport data, field data books and hard copies of the field maps used at the time of exploration should be maintained systematically
6. Addition of new information, corrections, and deletions should be incorporated on real time basis and all changes should be published on line only after authentication by experts

Note

- The recorded database should be maintained properly in both forms, hardcopy as well as softcopy.
- Database should provide the information/ instructions related to Thawing, retesting and regrowth.
- Precautions ad specific measures to te undertaken during Post-thaw regeneration should be mentioned in the database.
- Database should be updated regularly and timely for each cryostored sample.
- Data entry in the database should be done before the cryostorage to avoid any mismatch.

Points to remember

1. Database must be maintained with complete details.
2. Database entry must be done by expert/ competent person.
3. Always keep a copy of database at safe place outside the lab to avoid any mishappening and loss of data.

8. Citrus Field Genebank (FGB)

For *ex-situ* conservation of Citrus germplasm, field genebank (FGB) and cryogenebank are the only options. Field gene bank is required to establish and maintain plants in the field. Establishment of FGB for any perennial species requires lot of pre-planning considering the choice of propagation methods, planting sites, spacing of trees, disease and pest management measures, harvest and backup of germplasm. Overall objectives of any FGB are to maintain healthy and vigorous plants for conservation and utilization for crop improvement purposes with a minimum risk of loss or genetic fidelity. In FGB, the germplasm is maintained as live plants that undergo continuous growth and require regular maintenance. Regular monitoring for biotic and abiotic factors of plants is required and application of control measures is essential in order to maintain plants that are free of disease. The main advantages of Field gene banks are to provide an easy access to the plant genetic resources, for characterization, evaluation, research, education, training and utilization.

Presently most of the germplasm of *Citrus* species and allied genera is being maintained and conserved in field genebanks in several Citrus growing states of India. Approximately 2,000 accessions of indigenous and exotic germplasm are being maintained in approximately 20 small and large field genebanks in various agro-climatic zones of India. Some of the important field genebanks such as at Burnihat, Assam and Dhaulakuan, Sirmour, Himachal

Citrus Field Genebank at NRCC, Nagpur

Pradesh holding valuable *Citrus* germplasm have been completely declined (Singh and Singh, 2003). Other field genebanks which are older than 20-25 years, are also facing serious problems of some diseases like die-back or Citrus decline and others diseases. Therefore, the maintenance of Citrus field genebank is challenging task for curators and institutions. Field collections need to be replicated in Regional Field genebanks, these to be established in the different edaphoclimatic zones with all modern facilities and sufficient man power which is presently lacking in existing Citrus field genebanks. At least four Regional Field Repositories of Citrus need to be established spreading in Northeast, Northwest, Central and Southern parts of India.

8.1 Establishment of Citrus Field Genebank

Field genebank being established is for medium to long-term conservation and once established would become assest and also liability for the instituion. Therefore, this would require futuristic and meticulous planning. These basic questions are to be kept in mind before starting establishment of any FGB for Citrus:

1. How much land and resources are presently available and would be earmarked for future maintenance of FGB?
2. Is the FGB is being established purely for germplasm conservation or research on crop improvement would also be linked to it?
3. How many accessions are to be established in the FGB in short- to medium- and long-term?
4. How the germplasm would be procured for FGB-explorations or from other secondary sources?
5. How many primary and secondary collections would be established- with clear strategy on duplicate collections?
6. Do we need to have Screen house to establish plants and to provide back up for FGB and for this funds are available?
7. Is the staff especially Curator would be able to provide his/her services regularly till the period of establishment and beyond?
8. Most important need to learn lessons/consultations from already operational and also diminished/decayed FGBs to be come more vigilant, confident and technological stronger.

8.2. Selection of Land/Site for FGB

1. The agro-ecological and edapho-climaticconditions of the land being considered should preferablybe similar to the natural habitat of the species to be planted in order to reduce biotic and abiotic stresses
2. The site should be located at a place with minimum risk of natural and man-made catastrophes. It should be well protected preferably having boundary wall/fencing
3. For the Field Genebank (FGB) establishment, the land should be obtained or purchased legally to avoid any possession conflict in the future and to secure it for long-term basis
4. The land should be suited to irrigation and application of mulching, fertilizers and pesticides
5. It is essential to have a water source near the field genebank for irrigation and other agronomic and plant protection applications

Note:

- Sub-tropical climate is best for healthy growth and development of Citrus, therefore, be preferred.
- Citrus is extremely susceptible to hot wind, high humidity and frost.
- Some of mandarins, papeda and sweet orange cultivars/ genotypes as well as wild species need particular humidity and temperature or microclimate for establishment, vegetative growth and to acquire reproductive phase. Therefore, need to be grown in FGB situated in suitable climate and specific locations to meet these requirements.

Citrus FGB, PAU, Abohar

8.3. Preparation of Land

Materials required

a. Chemical for soil fumigation and sterilization

b. Cultivators

c. Fertilizer

d. Harrows

e. Irrigation facility

f. Land leveler

g. Spade

h. Tractor

1. Before starting any land operation ensure the soil and water quality of land. For optimal growth of Citrus, well-drained soil with the pH between 5.5 to 7.5 is ideal

2. If ground water or external water source is being used the TDS may not be more than 1000 ppm and pH should be in the range of 6.5-8.5

3. Citrus should be preferably grown between a temperature range of 13°C to 37°C and the temperature of soil should be preferably around 25°C for better root growth

4. The site should be well structured, fenced securely with ensured irrigation facility to make the plants safe and secure

5. Before planting, prepare the land by deep ploughing and leveling. In hilly areas mostly terrace planting is undertaken and terraces are made against the slopes

6. To avoid water logging drainage channels, need to be prepared around the field

7. Before planting, the land should be fumigated, solarized properly and made free of any weeds

Note:

- Soil and water testing is must before taking any land preparation activity.
- Proper security arrangement should be ensured for the safety of field genebank.
- Fencing should be strong enough to prevent animals from entering the field genebank.
- Land preparation is a crucial and most important aspect as plant growth would depend on it. Therefore, all care should be taken and no short cut be tried.

8.4. Acquisition of Germplasm

Materials Required

a. Labeling tag

b. Record book

c. Rootstocks

1. As per the preplanning, germplasm need to be procured at the right time from explorations, other FGBs and orchards etc.
2. In case of wild and polyembryonic species, seeds may be taken up as the propagule and in case any specific cultivar or elite type germplasm, scion may be collected and established to ensure true-to-type plants
3. All the collected samples should be labeled to avoid any mixing of samples and it is important to assign a unique accession number to each sample
4. The collected or exchanged propagules should be visibly healthy, disease-free and to be appropriately maintained till sowing or grafting is undertaken
5. In case of rootstocks, it is important to select them suitably so that they adapt well to the soil type and have as less impact on scion behavior as possible
6. Rootstocks should be kept ready before scion collected/procured and arrived to FGB

Note:

- The time period between collection, processing and transfer of the germplasm to FGB should be as short as possible to avoid any loss of material.
- Choice of propagule for planting may be thoroughly discussed and decided as per the biological status of genotype and future need of germplasm.

8.5 Nursery Establishment

1. Extract seeds from mature and healthy fruits immediately after arrival of fruits. If extracted seeds and scion are received, these need to be stored at ideal temperature and humidity
2. For the sowing of seeds primary or secondary nursery may be opted as per the need. For primary nursery beds prepared of fertile mixture or seedling trays filled with 1:1:1 mixture of sand: FYM/vermicompost : soil should be kept ready
3. For transferring plants directly to the field, seedlings may be raised in polythene bags till these attain height of 1-2 feet
4. Seed sowing for raised nursery production should be undertaken under partially shaded, netted area or in the screen house
5. Suitable rootstocks should be raised in nursery beds/polythene bags and for vegetative propagation air layering or T- budding may be attempted
6. Rootstock must be compatible to scion and it should be collected from true- to-type and disease free and healthy mother plant

Note

1. Establishment of germplasm in nursery is highly critical and needs thorough knowledge of propagation method and nursery techniques.
2. Procured germplasm should be established using appropriate method and skill to achieve maximum success after establishment.
3. In case where true-to type plants are to be raised, suitability of rootstock and right grafting technique should be used.

FGB of exotic collections of Citrs

8.6 Layout Designing

Citrus is a large genus with diverse species having different plant types, canopies, growing habits, seasonality, reproductive biology, flowering and fruiting time etc. Various species of Citrus for the purpose of convenience have been grouped in to different types like, mandarins, oranges, lime and lemon, pummelo and grapefruit, papeda and other wild species. Based on their habits and various phenotypic and physiological characters there is a need to establish separate FGB blocks for each group of Citrus germplasm. This would bring uniformity within the block, and help in comparing various parameters, ease of data collection and also handling of germplasm. The layout should be designed in such a way that wild species, cultivmated species and rootstocks should be planted separately.

Materials Required

a. Compass

b. Measuring tape

c. Planting board

d. Pencil/ Pen

e. Record book

1. Overall layout for FGB and separate layouts for the individual block need to be meticulously planned as each citrus group has different agronomic and field requirements

2. A field map preferably digital form should be designed and prepared clearly indicating the exact location of each plant and accession in a block

3. The position of accessions and spacing between each plant should be appropriate and as per the canopy of particular citrus species.For FGB spacing of plants may be reduced from what is recommended for the commercial orchards

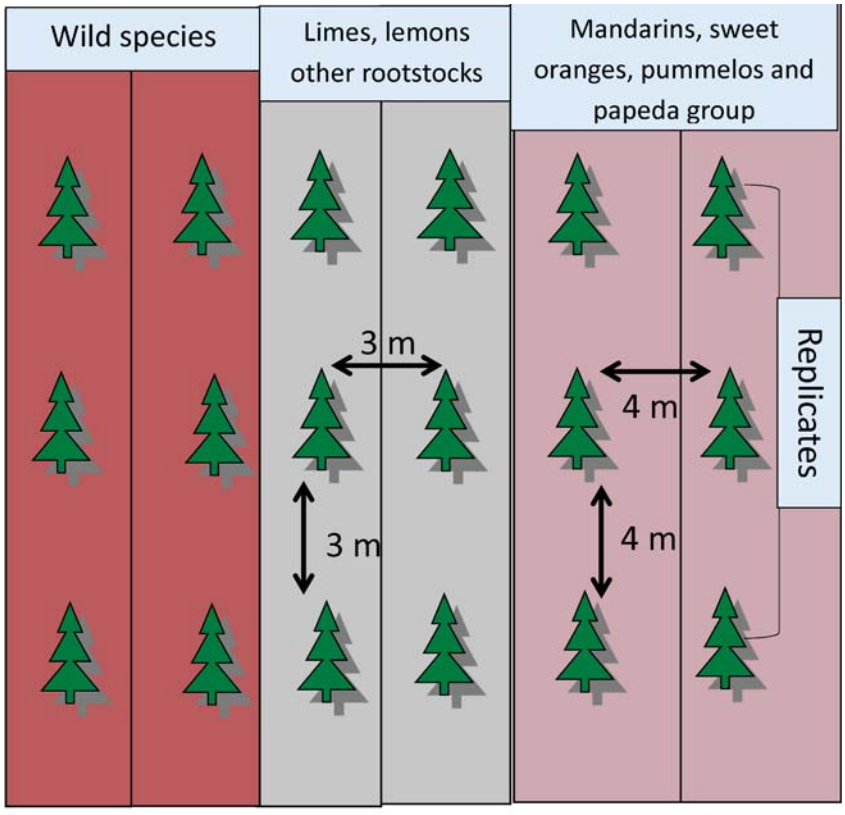
4. For mandarins, sweet oranges and pummelos normal spacing may be 4 m ×4 m while for limes/lemons and other wild species spacing should be 3 m × 3 m. Similarly for hilly terranes dense planting may be adopted as more aerial space is available

5. It is important to label each and every plant correctly for effective monitoring and other uses. Digital labelling using Bar code/ QR code/ microchips may be used to facilitate tagging of each tree

6. Layout should be handy and available to all staff dealing with FGB. Duplicate copy of layout should be safely kept in records and as a digital document.

Note

- The data of each accession should be recorded in digital as well as printed form.
- The field layout should be precise.
- Spacing between plants/ accessions should be decided keeping in view the future growth of plants.



Layout for Citrus Field Genebank

8.7 Field Establishment

Materials Required

a. Farm equipment
b. Meter scale
c. Manures and fertilizers
d. Planting board
e. Spade
f. Trowel

1. Planting is an important activity of FGB management, the nursery grown plants should be planted in the field at appropriate time (July-September and February to April, preferably)
2. For planting, healthy, disease free, seedlings/grafted plants of minimum 1.5 to 2 feet height should be considered
3. The pit 1×1×1 m should be dug about one month before planting for proper solarization
4. Well-rotted FYM (15-20 kg) and other fertilizers (single Super Phosphat) should be properly mixed with soil for filling the pit
5. Remove the polythene bag carefully to protect the root system and main stem. Ensure that plant is placed at the centre of pit
6. While filling back the pit it is to be ensured that stem of seedling especially bud union is not in the soil and soil is pressed well or watered to avoid any air pockets
7. After planting the seedlings/grafts should be adequately watered to avoid any moisture stress
8. It is important to maintain an adequate number of plants (minimum three plants per accession) to exhibit genetic diversity and to ensure safety of conserved germplasm

Note

- It is important to make all prior arrangements before undertaking transplanting of plants in the field.
- Timing of transplanting should be appropriately fixed as low temperatures (below 4°C) and high temperatures (above 40°C) are unfavorable for young plants
- Watering of young plants should be carefully monitored to ensure no water deficit or logging.
- Plant health should be monitored regularly.

8.8. Field Management

Materials required

a. Fertilizers

b. Field and farm equipment's

c. Pesticides

d. Pruning shears

e. Secateurs

f. Weedicides

Field management of Citrus plant under FBG

1. The soil should be irrigated whenever required. Water logging should be avoided as it may affect Citrus plants adversely
2. Cultural practices like weed control, pruning, fertilizer and pesticides application should be done timely and as per the requirement
3. FGB Curator should be attentive about the water supply and soil nutrition as Citrus is susceptible to water stress/logging and soil-borne diseases
4. Regularly monitor the accessions for deficiency, disease out breaks and major diseases like Die-back etc. in order to undertake control measures timely
5. Keep germplasm of rare and important accessions as potted/field plants in the screen house as a backup
6. Regularly regenerate and multiply the important germplasm for safe conservation and maintenance. Maintain duplicate accessions in other FGBs for safety purposes
7. A secure backup of important germplasm in the clonal repository, cryogenebank and *in vitro* genebank is must to achieve safe and holostic conversation of citrus germplasm

Note

- Maintain general hygiene and cleanliness in the FGB and timely remove the infested branches and fruits from plants as well as from the ground.
- One should be well aware of the diseases and pests with relation to Citrus so as to treat the plants effectively as and when required.
- The backup data of FGB plants should be maintained and updated regularly.
- Complimentary conservation is the best strategy for conserving Citrus being a vast and diverse genus, therefore, besides FGB it is better keep one set of germplasm in cryogenebank.

8.9. Characterization, Evaluation and Documentation

Materials required

a. Computer/Laptop

b. Data record book

c. Facility of testing acidity, phenols, sugars etc.

d. Measuring scale

e. Micrometer

f. Refractometer

g. Standard descriptor

h. Statistical software

i. Verner's caliper

Citrus fruit for Characterization

1. Characterize Citrus field collections once these attain the maturity (completion of gestation period) using standard descriptors

2. Germplasm should be characterized for morpho-physico-chemical and species-specific traits to understand existing diversity and its genotypic value

3. Record the characterization data for regular 3 years (leaving first year of attaining maturity) and statistically analyze the data to add value to the germplasm

4. Based on characterization data select the germplasm and specific traits to be further evaluated

5. Characterize and evaluate the citrus germplasm based on defined groups to draw a logical conclusion out of collected data.

6. Specific and desirable traits important for utilization of germplasm need to be evaluated. These traits may be vegetative, reproductive, yield related, quality related, biotic and abiotic stress etc.

7. The evaluation data should be statistically analyzed and used for enhancing value of germplasm for crop improvement purposes

8. Document passport data, field establishment data, field management data, characterization and evaluation data in an appropriately designed database

9. Regularly update the data of FGB collections, share it and utilize for various purposes

10. Take backup of documented data at regular intervals and keep it as printed as well as electronic form at least at two locations for safety purpose

Note

- The characterization and evaluation data should be taken on plants which attain maturity at least one year earlier.
- Characterize and evaluate germplasm within the groups for right conclusion and utility.
- Characterization and evaluation data should be taken at least for 3 years and maximum for 5 years before coming to any conclusion.
- An FGB information management system should be developed and maintained for the best utilization of germplasm and its long-term sustainability.

9. Suggested Readings

1. Bhag Mal, Ramamani YS and Ramanatha Rao V, editors (2001). Proceedings of the First Annual Meeting of the Project, 'Conservation and Use of Native Tropical Fruit Species Biodiversity in Asia' Pattaya, Thailand, 6-9 February 2001. IPGRI, South Asia Office, New Delhi, India.

2. Chaudhury R and Malik SK (2017) Cryopreservation of Plant Species: Practical Approaches from Handling to Cryobanking. ICAR-National Bureau of Plant Genetic Resources, New Delhi. pp 52.

3. Chaudhury R and Malik SK (2016). Expanding applications of cryobanking: meeting challenges for effective long-term storage. *Indian J. Plant Genet. Resour.* 29(3): 303-306.

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

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

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

7. Kameswara Rao N and Bramel PJ (eds.). (2000). Manual of Genebank Operations and Procedures. Technical Manual no. 6. Patancheru 502 324, Andhra Pradesh, India: International Crops Research Institute for the Semi-Arid Tropics. pp. 15-17.

8. Kaur S, Malik SK, Choudhary R, Rohini MR, Chaudhury R and Kumar R (2019) Morphological characterization of pummelo germplasm collected from different parts of India. *Indian J. Hort*. 76 (1), 16-22.

9. Kumar S, Malik SK, Chaudhury R and Bhat SR (2014) A new wild type of Citron (*Citrus medica* L., Rutaceae) identified through morphology and psbM-trnD spacer region of chloroplast DNA. *Tree Genetics & Genomes*, DOI 10.1007/s00468-014-1022-1.

10. Malik SK, Choudhary R, Kaur S, Chaudhury R and Pritchard HW (2020) Storage behavior and cryopreservation of *Citrus cavaleriei*, an endangered, cold-resistant species of northeast India with exceptionally large seeds. *CryoLetters*, 41(5), 281-290.

11. Malik SK and Chaudhury R (2019) Cryopreservation Techniques for Conservation of Tropical Horticultural Species Using Various Explants. In: Rajasekharan P., Rao V. (eds) *Conservation and Utilization of Horticultural Genetic Resources*. Springer, Singapore, P 579-594.

12. Malik SK, Singh IP, Kumar S and Chaudhury R (2017) Revisiting Citrus genetic resources of Northeast India. In: *Doubling Farmers Income through Horticulture*. Eds. K.L. Chadha et al., Daya Publishing House, New Delhi. pp 947-954.

13. Malik SK, Chaudhury R and Singh IP (2015) Management of Citrus genetic resources in India: approaches, applications and achievements. *Acta Hortic*. 1101: 39-46. (DOI 10.17660/*Acta Hortic*.2015.1101.7)

14. Malik SK, Chaudhury R, Kumar S, Dhariwal OP and Bhandari DC. (2014). Citrus Genetic Resources in India: Present Status and Management. National Bureau of Plant Genetic Resources, New Delhi, India. p.184.

15. Malik SK, Chaudhury R and Pritchard HW (2012) Long-term, large scale banking of *Citrus* species embryos: comparisons between cryopreservation and other seed banking temperatures. *CryoLetters*: 33 (6), 453-464.

16. Malik SK, Rohini MR, Kumar S, Choudhary R, Pal D and Chaudhury R (2012). Assessment of genetic diversity in sweet orange [*Citrus sinensis* (L.) Osbeck] cultivars of India using morphological and molecular markers. *Agricultural Research (Springer)*: DOI 10.1007/s40003-012-0045-3.

17. Malik SK, Uchoi A, Kumar S, Choudhary R**,** Pal D, Kole PR, Chaudhury R and Bhat KV (2012) Molecular characterization of *Citrus macroptera* Montr. (Satkara): an endangered wild species from Northeastern India. *Plant Biosystems* DOI:10.1080/11263504.2012.751063.

18. Malik SK**,** Susheel Kumar, IP Singh, OP Dhariwal and Chaudhury R (2013) Socio-economic importance, domestication trends and *in situ* conservation of wild *Citrus* species of Northeast India. *Genetic Resources and Crop Evolution*: DOI 10.1007/s10722-012-9948-x

19. Malik SK, Kumar S, Chaudhury R and Nair KN (2012) Conservation strategies for Citrus genetic resources. Lead Paper In: National Dialogue on *Citrus Improvement, Production and Utilization.* Souvenir and Abstracts, Indian Soc. of Citriculture and NRC Citrus, Nagpur, Feb. 27-29, 2012. PP 24-32.

20. Malik SK, Susheel Kumar, IP Singh, OP Dhariwal and Chaudhury R (2012). Socio-Economic Importance of Wild and Semi-wild Citrus species of Northeast India. In:

Shivankar, V.J. and Singh, I.P. (eds). *Citrus Biodiversity*. Indian Society of Citriculture (ISC), Nagpur, Maharashtra. pp 172 - 177

21. Malik SK, Susheel Kumar, Digvender Pal, Ravish Choudhary, Ajit Uchoi, Rohini M R and Chaudhury R (2012). Cryopreservation and In vitro Conservation of Citrus Genetic Resources. In: Shivankar, V.J. and Singh, I.P. (eds). *Citrus Biodiversity*. Indian Society of Citriculture (ISC), Nagpur, Maharashtra. pp 156 – 168.

22. Malik SK and Chaudhury R (2006). The cryopreservation of embryonic axes of two wild and endangered *Citrus* species. *Plant Genetic Resources: Characterization & Utilization (UK):*4 (3): 204-209.

23. Malik SK, Chaudhury R, Dhariwal OP and Kalia RK (2006). Collection and characterization of *Citrus indica* Tanaka and *C. macroptera* Montr.: wild endangered species of northeastern India. *Genetic Resources and Crop Evolution*: 53 (7): 1485-1493.

24. Malik SK, Chaudhury R and Kalia RK (2003). Seed storage behaviour and cryopreservation of tropical fruit species. In : *In Vitro Conservation and Cryopreservation of Tropical Fruit Species*. IPGRI Office for South Asia, New Delhi, India/ NBPGR, New Delhi, India. Pp 175-190.

25. Pal D, Malik SK, Kumar S, Choudhary R, Sharma KC and Chaudhury R (2013) Genetic Variability and Relationship Studies of Mandarin (*Citrus reticulata* Blanco) Using Morphological and Molecular Markers. *Agricultural Research (Springer)*: 2: 236-245.

26. Pritchard HW, Nadarajan J, Ballesteros D, Thammasiri K, Prasongsom S, Malik SK, Chaudhury R, Kim HH, Line L, Lie WQ, Yange XY, Popova E (2017) Cryobiotechnology of tropical seeds – scale, scope and hope. I International Symposium on Tropical and Subtropical Ornamentals, Acta Horticulturae 1167.ISHS2017.DOI 10.17660/ *Acta Hortic*. 2017.1167.6.

27. Rohini MR, Malik SK, Choudhary R, Kaur S, Uchoi A and Chaudhury R (2016) Storage behavior and cryopreservation studies in Indian rough lemon (*Citrus jambhiri*):a promising rootstock for long-term conservation. *Turk J Agric For* 40: 865-873.

DBT Sponsored Project:

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