Shaping human resources to harness the global gene pool for sustainable agriculture

World Bank - ICAR Funded
NATIONAL AGRICULTURAL HIGHER EDUCATION PROJECT
Centre for Advanced Agricultural Science and Technology (CAAST) on
Genomics Assisted Crop Improvement and Management

Training Manual

Plant Genetic Resources Management and Utilization

ICAR - National Bureau of Plant Genetic Resources
New Delhi - 110012, INDIA

NAHEP Sponsored

Short Term Training Programme

on

PLANT GENETIC RESOURCES MANAGEMENT AND UTILIZATION

September 30 - October 11, 2019

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Germplasm diversity in seed genebank; Encapsulated shoot tip; *Gossypium cernuum* boll; Inside view of Genebank LTS module; *Carpophilus truncatus* pest; National Gene Bank; Cryo Genebank; Post Entry Quarantine of exotic soybean; Pop corn type maize from Nagaland; Grouping of rice germplasm accessions based on 50K SNP chip; *In vitro* genebank; SEM image of *Hibiscus lunarifolius* seed; Proliferating shoot meristems for *In vitro* conservation; SEM image of *Hibiscus lunarifolius* seed trichome; *Colletotrichum lindemuthianum* incidence on chilli

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About NAHEP-CAAST at IARI, New Delhi

Centre for Advanced Agricultural Science and Technology (CAAST) is a new initiative and student centric subcomponent of World Bank sponsored **National Agricultural Higher Education Project (NAHEP)** granted to The Indian Council of Agricultural Research, New Delhi to provide a platform for strengthening educational and research activities of post graduate and doctoral students. The ICAR-Indian Agricultural Research Institute, New Delhi was selected by the NAHEP-CAAST programme. NAHEP sanctioned Rs 19.99 crores for the project on "**Genomic assisted crop improvement and management**" under CAAST programme. The project at IARI specifically aims at inculcating genomics education and skills among the students and enhancing the expertise of the faculty of IARI in the area of genomics.

Objectives:

1. **To develop online teaching facility and online courses for enhancing the teaching and learning efficiency, and scientific communications skills**
2. **To develop and/or strengthen state-of-the art next-generation genomics and phenomics facilities for producing quality PG and Ph.D. students**
3. **To develop collaborative research programmes with institutes of international repute and industries in the area of genomics and phenomics**
4. **To enhance the skills of faculty and PG students of IARI and NARES**
5. **To generate and analyze big data in genomics and phenomics of crops, microbes and pests for genomics augmentation of crop improvement and management**

IARI's CAAST project is unique as it aimed at providing funding and training support to the M.Sc. and Ph.D. students from different disciplines who are working in the area of genomics. It will organize lectures and training programmes, and send IARI students and covering students from several disciplines. It will provide opportunities to the students and faculty to gain international exposure. Further, the project envisages developing a modern lab named as **Discovery Centre** that will serve as a common facility for students' research at IARI.

Core-Team Members:

S.No.	Name of the Faculty	Discipline	Institute
1.	Dr. Ashok K. Singh	Genetics	ICAR-IARI
2.	Dr. Vinod	Genetics	ICAR-IARI
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Acknowledgments

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4. **National Coordinator, NAHEP, ICAR, New Delhi**
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11. **Staff & Students, ICAR-NBPGR, New Delhi**

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NEW DELHI-110012

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अधिष्ठाता एवं संयुक्त निदेशक (शिक्षा) – कार्यकारी
Dr. (Mrs.) Rashmi Aggarwal
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FOREWORD

Plant Genetic Resources (PGR) comprise the sum total of the genetic diversity accumulated through the years of evolution. These PGRs are the raw material that enabled farming communities and agriculture to evolve in order to combat food security, poverty alleviation and sustainable livelihood. Efficient management and sustainable utilization of the PGRs is the key to all agricultural development and prosperity at global level. Recent developments have changed the PGR perspective from 'common heritage' to 'sovereign rights'. During the last two decades, a number of treaties, laws and conventions have changed the way PGRs are collected, evaluated, conserved, exchanged and utilized.

The ICAR-National Bureau of Plant Genetic Resources, New Delhi is mandated for the management and promotion of sustainable use of plant genetic and genomic resources of various agri-horticultural crops, to carry out related research, co-ordination, capacity building in PGR management; and policy issues governing access and benefit sharing of their use; and molecular profiling of varieties of agri-horticultural crops. The institute has state-of-the-art facilities and trained scientific strength in the area of plant genetic resources research.

The present training on 'Plant Genetic Resources Management and Utilization' under the Centre for Advanced Agricultural Science and Technology of NAHEP is specially conceived by the ICAR-National Bureau of Plant Genetic Resources to impart a comprehensive knowledge about efficient management and utilization of plant genetic resources to post-graduate students of NARES system who are the probable leaders for future agricultural development. I am sure that the training will be highly beneficial to the post-graduate and Ph.D. students in the area of Plant Genetic Resources.

(Rashmi Aggarwal)

September 27, 2019

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PREFACE

Plant Genetic Resources management encompasses a multilevel domain of activities at scientific, ecological, social and policy levels. Conservation and sustainable utilization of the plant genetic resources has drawn utmost attention at global level due to various threats posed on them. India being a mega-biodiversity centre with varied climatic conditions, systematic management and therefore its utilization is a challenging task. ICAR-National Bureau of Plant Genetic Resources (ICAR-NBPGR) is a nodal organization established in 1976, with a national responsibility to plan, execute and coordinate all plant genetic resources' management and utilization activities viz., exploration and collection; exchange; quarantine; characterization and evaluation; DNA fingerprinting, genomic resources' generation and GM detection; ex situ, in situ and in vitro conservation of germplasm.

Significant achievements have been made in India with respect to plant genetic resource conservation, which may not be in place at the same time in many developing countries. ICAR-NBPGR has the mandate of conserving the plant genetic resources with its national base collection at New Delhi, linked to several medium-term collections in a system-wide approach in the country. Bureau has also been vested with the authority to issue Import Permit and Phytosanitary Certificate and conduct quarantine checks on all seed materials and plant propagules (including transgenic material) introduced from abroad or exported for research purpose. It also provides Human Resource Development in all spheres of PGR management and periodical reorientation thereof, to the emerging scientific and technological developments from time to time.

Centre for Advanced Agricultural Science and Technology (CAAST) is a student centric subcomponent of the World Bank sponsored National Agricultural Higher Education Project (NAHEP) granted to IARI to provide a platform for strengthening educational and research activities of post-graduate and doctoral students. With a view to impart the knowledge of plant genetic resource conservation and management, the PG School, IARI, has entrusted the ICAR-NBPGR, New Delhi with the responsibility to organize the training for the students of ICAR recognised State Agricultural Universities (SAUs) and UGC recognised Universities in the field of Management of Plant Genetic Resources and Conservation. The major objective of the training programme is to generate awareness among the student about the importance of plant genetic resources and their management for sustainable development of agriculture and future food security. The training manual is an excellent source of information to update the student's skills in this area. The theory and the practical exercises including the hands-on-training on the topics related to the entire activities in PGR are contained in this manual for the benefit of the students. The contributors of different chapters are eminent scientists and experts in their respective fields. We are extremely thankful to them for sparing their valuable time to write respective chapters in spite of their busy schedule.

Kuldeep Singh
Veena Gupta
Era Vaidya Malhotra

NAHEP – CAAST

Short Training Programme

Plant Genetic Resources Management and Utilization

30 September – 11 October, 2019

Training Schedule

Date	Time	Topic/Activity	Speaker
September 30 Monday	10:30 – 11:30 am	Inauguration	**Dr. K. C. Bansal** Senior Fellow, TERI-Deakin Nanobiotechnology Centre
	11:30 – 12:00 noon	**Tea**	
	12:00 – 1:00 pm	Exploration & germplasm collection: status, priorities and future thrust	**Dr. S. P. Ahlawat** ICAR-NBPGR
	1:00 – 2:00 pm	**Lunch**	
	2:00 – 5:30 pm	**Practical:** From field to the herbarium of cultivated plants: 'the hidden garden of plant genetic resources'	**Dr. Anjula Pandey** ICAR-NBPGR
October 1 Tuesday	9:30 – 10:30 am	Avenues and opportunities for PGR utilization: and industry perspective	**Dr. Deepak Prem** Bayer Crop Science
	11:00 – 12:00 noon	International and national regulations governing the use of PGR	**Dr. Pratibha Brahmi** ICAR-NBPGR
	12:00 – 1:00 pm	PGR Informatics	**Dr. Sunil Archak** ICAR-NBPGR
	1:00 – 2:00 pm	**Lunch**	
	2:00 – 5: 30 pm	**Practical:** PGR Informatics	**Dr. Sunil Archak** and **Mr. Rajeev Gambhir** ICAR-NBPGR
October 2 Wednesday		Visit to Issapur farm	**Dr K. K. Gangopadhyay**, **Dr. V S Meena,** **Dr. Kuldeep Tripathi** and **Era Vaidya Malhotra** ICAR-NBPGR
October 3 Thursday	9:30 – 10:30 am	PGR management and utilization: an ethos of Indian traditional agriculture and civilizations	**Dr A. K. Singh** Former Head, DGC, ICAR-NBPGR
	11:00 – 12:00 noon	Characterization and evaluation of PGR	**Dr K. K. Gangopadhyay** ICAR-NBPGR
	12:00 – 1:00 pm	Pre-breeding for effective use of PGR	**Dr. V K Sharma** ICAR-NBPGR
	1:00 – 2:00 pm	**Lunch**	

	2:00 – 5:30 pm	**Practical**: Biochemical and phytochemical evaluation of germplasm	**Dr Rakesh Bhardwaj,** **Dr Archana Raina** ICAR-NBPGR
October 4 Friday	9:30 – 10:30 am	Role of ICAR-NBPGR in PGR management	**Dr. Kuldeep Singh,** Director, ICAR-NBPGR
	11:00 – 12:00 noon	Germplasm collecting: logistics and tactics	**Dr. K C Bhat** ICAR-NBPGR
	12:00 – 1:00 pm	PGR – Conservation and Management	**Dr. Veena Gupta** ICAR-NBPGR
	1:00 – 2:00 pm	**Lunch**	
	2:00 – 5:30 pm	**Practical**: Conservation procedures for seed gene banks	**Dr. Anjali Kak Koul** and **Dr. Sherry Rachel Jacob** ICAR-NBPGR
October 5 Saturday	9:30 – 10:30 am	Introduction and exchange of PGR	**Dr Vandana Tyagi** ICAR-NBPGR
	11:00 – 12:00 noon	Plant tissue culture interventions for management of plant genetic resources	**Dr. Anuradha Agrawal** ICAR-NBPGR
	12:00 – 1:00 pm	Principles and applications of cryopreservation	**Dr. Rekha Chaudhury** ICAR-NBPGR
	1:00 – 2:00 pm	**Lunch**	
	2:00 – 5:30 pm	**Practical**: Strategies for i*n vitro* conservation and cryopreservation of germplasm	**Dr. Sangita Bansal,** **Dr. Vartika Srivastava** **Dr. Era Malhotra** and **Dr. Gowthami R.** ICAR-NBPGR
October 6 Sunday		**Visit to Biodiversity Park**	**Dr. Veena Gupta,** **Dr. Anjali Kak Koul,** **Dr. Vandana Tyagi** and **Dr. Sandhya Gupta** ICAR-NBPGR
October 7 Monday	9:30 – 10:30 am	Some important plant resources from Western Ghats	**Dr. S R Yadav** Shivaji University, Kolhapur
	11:00 – 12:00 noon	Principles of plant quarantine and national quarantine setup in India	**Dr. S C Dubey** ICAR-NBPGR
	12:00 – 1:00 pm	Quarantine procedures for exchange of PGR	**Dr. Kavita Gupta** ICAR-NBPGR
	1:00 – 2:00 pm	**Lunch**	
	2:00 – 5:30 pm	**Practical**: Detection and identification of pests in exotic germplasm including transgenics	**Dr. Celia Chalam** **Dr. Kavita Gupta** **Dr. Mool Chand** **Dr. Jameel Akhtar** **Dr. Bharat Gawade** and **Dr. Pradeep Kumar** ICAR-NBPGR
October 8 Tuesday	**HOLIDAY**		

October 9 Wednesday	9:30 – 10:30 am	Characterization of PGR for genomics assisted crop improvement	**Dr. Swarup Parida** NIPGR, New Delhi
	11:00 – 12:00 noon	Role of genomics in PGR management	**Dr Ambika Gaikwad** ICAR-NBPGR
	12:00 – 1:00 pm	DNA fingerprinting of crop varieties	**Dr. Mukesh Rana** ICAR-NBPGR
	1:00 – 2:00 pm	**Lunch**	
	2:00 – 5:30 pm	**Practical**: Molecular techniques for germplasm characterization	**Dr. Lalit Arya, Dr. Manjusha Verma** and **Dr. Rakesh Singh** ICAR-NBPGR
October 10 Thursday	9:30 – 10:30 am	Ecological niche perspectives on PGR	**Dr. R Umashankeer** GKVK, Bengaluru
	11:00 – 12:00 noon	Biosecurity at CGAIR centres with special reference to ICRISAT	**Dr. K. S. Varaprasad** Former Director, ICAR-IIOR, Hyderabad
	12:00 – 1:00 pm	Advances in PGR evaluation	**Dr Ashok Kumar** ICAR-NBPGR
	1:00 – 2:00 pm	**Lunch**	
	2:00 – 3:00 pm	Visit to NBPGR Museum	**Dr. Chithra Pandey,** and **Dr. Bharat Gawade** ICAR-NBPGR
	3: 00 – 5:30 pm	Visit to NGB, *In vitro* Genebank and Cryobank	**Dr Neeta Singh** ICAR-NBPGR
October 11 Friday	10:00 – 1:00	Visit to IARI Phenomics facility and Phytotron facility	**Dr Veena Gupta,** and **Dr Era Malhotra** ICAR-NBPGR
	1:00 – 2:00 pm	**Lunch**	
	2:00 pm – 3:30pm	Valedictory Function	**Dr K. V. Prabhu** Chairman, PPV&FRA, New Delhi and **Dr. R. C. Agrawal** National Director, NAHEP

CONTENTS

1
Role of ICAR-NBPGR in PGR Management

Kuldeep Singh* and Kavita Gupta
ICAR-National Bureau of Plant Genetic Resources, New Delhi-110012
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The National Bureau of Plant Genetic Resources (ICAR-NBPGR) was established by the Indian Council of Agricultural Research (ICAR) in 1976 with its headquarters at New Delhi. The chronology of events leading to the present day ICAR-NBPGR dates back to 1905 when Botany Division was established under the then Imperial Agricultural Research Institute. ICAR-NBPGR has been given the mandate to act as a nodal institute at the national level for acquisition and management of indigenous and exotic plant genetic resources (PGR) for agriculture, and to carry out related research and human resources development for sustainable growth of agriculture. The Bureau is also vested with the authority to issue Import Permit and Phytosanitary Certificate and conduct quarantine checks in seed material and vegetative propagules (including transgenic material) introduced from abroad or exported for research purposes. Besides having a 40 ha experimental farm at Issapur village (about 45 km west of Pusa Campus), the Bureau has a strong national network comprising Regional Stations/ Base Centers and ICAR Institutes/ SAUs that provide access to representative agro-ecological situations in the country.

Mandate: The mandate includes management and promote sustainable use of plant genetic and genomic resources of agri-horticultural crops and carry out related research; coordination of capacity building in PGR management and policy issues governing access and benefit sharing of their use, and molecular profiling of varieties of agri-horticultural crops and GM detection technology research.

ICAR-NBPGR has its headquarters in New Delhi that hosts the second largest genebank in the world. The operations are administered by Divisions of Plant Exploration and Germplasm Collection, Germplasm Evaluation, Germplasm Conservation, Genomic Resources and Plant Quarantine in addition to the Units of Germplasm Exchange and Tissue Culture and Cryopreservation. ICAR-NBPGR has the network of 10 Regional Stations covering different agro-climatic zones to carry out PGR activities including collection, characterization, evaluation and maintenance of various crops as mentioned below:

- ***Shimla (Himachal Pradesh):*** Established in 1960; temperate crops.
- ***Jodhpur (Rajasthan):*** Established in 1965; agri-horticultural crops germplasm of arid and semi-arid zones.

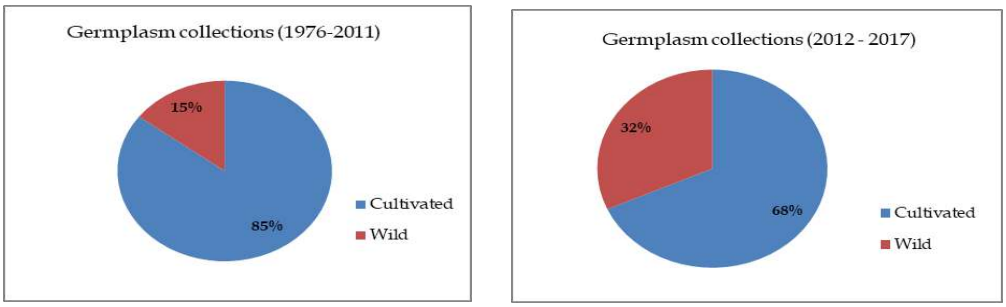
- ***Thrissur (Kerala):*** Established in 1977; agri-horticultural crops germplasm of southern peninsular region with particular emphasis on spices and plantation crops.
- ***Akola (Maharashtra):*** Established in 1977; agri-horticultural crops germplasm of central India and Deccan Plateau.
- ***Shillong (Meghalaya):*** Established in 1978; agri-horticultural crops germplasm of north-eastern region including Sikkim and parts of north Bengal.
- ***Bhowali (Uttarakhand):*** Established in 1985; agri-horticultural crops germplasm of sub-temperate region.
- ***Cuttack (Odisha):*** Established in 1985; agri-horticultural crops germplasm of eastern peninsular region with main emphasis on rice germplasm.
- ***Hyderabad (Telangana):*** Established in 1985; Quarantine clearance of agri-horticultural crops germplasm of Telangana, Andhra Pradesh and adjoining areas.
- ***Ranchi (Jharkhand):*** Established in 1988; germplasm of tropical fruits and other field crops of Bihar, eastern Uttar Pradesh, Jharkhand and West Bengal.
- ***Srinagar (Jammu & Kashmir):*** Established in 1988; agri-horticultural germplasm of temperate crops.

PGR are conserved in the form of seeds, vegetative propagules, tissue/ cell cultures, embryos, gametes, etc. in the Genebank. The National Genebank facility commissioned in 1997 has 12 long term storage modules, each with a storage capacity of 50,000 to 76,000 samples depending upon the size of seeds. Its cryopreservation facility contains six liquid nitrogen storage tanks (cryo-tanks), each having a capacity to hold 1,000 litres of liquid nitrogen. These six cryo-tanks have a total capacity to store 0.25 million samples. Thus, the National Genebank has a total capacity to store 0.85 to 1.25 million samples. This is one of the most modern Genebanks of the world.

The Bureau not only conserves PGR safely to meet the needs of future generations, but also provides these to the nation's crop improvement programmes to sustain continued advances in agricultural productivity and stabilize production. The Bureau works in close collaboration with several international institutes/ organizations through memoranda/ work plans developed under bilateral/ multilateral agreements. It exchanges plant germplasm with over 100 countries. The Bureau is gradually developing and strengthening the national plant genetic resources system by linking up the National Base Collection (kept under long-term storage at ICAR-NBPGR with National Active Germplasm Sites (NAGS) responsible for different crops where germplasm collections are evaluated and multiplied under field conditions backed by medium-term storage facilities. The various activities and achievement of the Bureau are presented here, briefly.

PGR Exploration and Collection

- To develop new varieties in various agri-horticultural crops for farmers suitable to different agro-climatic conditions, new germplasm/ parent material with desired traits or genes is a continuous requirement of the plant breeders. Therefore, periodically such germplasm is collected by ICAR-NBPGR Scientists in collaboration with crop-based institutes of ICAR. Also, the trait-specific germplasm of various crops has been collected from diversity-rich spots (including the difficult unreached areas in different parts of the country).
- The institute has so far undertaken 2644 explorations and collected about 2.67 lakhs accessions of crop species and their wild relatives.
- **Focus on North-East and rescue missions**: In total, 165 explorations undertaken and a total of 9,698 accessions (cultivated- 6,622, wild- 3,076) collected. 45 exploration trips to North-Eastern Hill Region and five rescue missions to natural calamity affected areas of Uttarakhand were undertaken. Systematic explorations have been conducted in the remotest parts of the country including Mon district of Nagaland; Anjaw, Changlang and Tirap districts of Arunachal Pradesh; and Great Nicobar. Disturbed/insurgency-prone areas such as Bastar region in Chhatttisgarh; Gadchiroli in Maharashtra; and West Medinipur in West Bengal resulting in collection of 858 landraces of different agri-horticultural crops. Gap analysis, geo-referencing and diversity distribution mapping completed in nine crops.
- **Priority was given to the crop wild relatives (CWR)** which resulted in collection of 576 unique accessions which resulted in a significant increase in the share of wild species (32%) in the total collection.



Increased focus on CWR in germplasm collecting

PGR Exchange

- ICAR-NBPGR is the nodal agency for import and export of all PGR for research purpose, adhering to guidelines of National Biodiversity Act, 2002.
- ICAR-NBPGR is instrumental in introduction of several new crops in India such as soybean, sunflower, kiwi, tree tomato, oil palm, jojoba, guayule, hops etc. and aromatic

plants like rose geranium which are getting popular in Himalayan states, Uttarakhand and HP.

- More than 10, 000 accessions of indigenous and introduced germplasm are supplied annually to the researchers throughout the country.
- Exchange carried out with >100 countries and CGIAR institutes under bi-or multi-lateral agreements. Annually, ~25,000 accessions of PGR and ~75,000 samples of trial material (primarily from CG Institutes) are introduced into India for use in crop improvement programmes. Till date, about 6.5 lakh germplasm accessions of various crops including the transgenic planting material have been introduced/ imported into the country. This is now facilitated through online application for import permit (http://www.nbpgr.ernet.in/gep/). These introductions have been used both for direct release as varieties and in crossing programme as parents. Hundreds of such examples are available and some classical examples of use of exotic germplasm in varietal development is presented in Table 1.

Table 1: Use of introduced exotic germplasm in development of new varieties

Crop	Variety/Hybrid	Exotic Source
Rice	Annada	MTU-15 / Yaikaku Nantoku (China)
Barley	Clipper, Alfa 93, Rekha	Introduction from Australia
Chickpea	C104, L550	Rabat (Morocco)
	BG261, BG244	P9847 (Russia)
	BG267	USA 613 (USA)
Tomato	Kashi Hemant	Sel 18 / Flora Dade
	Punjab Kesari	EC 55055 / Punjab Tropics
	Punjab Chhuhara	EC 55055 / Punjab Tropics
Bottle gourd	Kashi Ganga	IC92465 / DvBG-151
Chilli	Co3	CA 856 (Srilanka)
Pea	Uttra	EC109185 / HFP4
Carrot	Pusa Yamdagni	EC 9981 / Nantes
Sweet potato	Pusa Sunehari	Porto Blanco / Wanrap) //Australian Cannanes (USA)
Squash	Pusa Alankar	(EC 27050 / Selection No. PL.8)
Cauliflower	Pusa Snowball-1	EC 12012 / EC12013
Cowpea	Aseem	Pusa Phalguni / EC 21622 (Philippines)
	Rituraj	Pusa Dofasali / EC 26410 (Mexico)
	Pusa Dofasali	Pusa Phalguni / EC 21622 (Philippines)
Cluster bean	RGS 936	EC 248 / RGC 401

Not only the exotic germplasm introduced from other countries has helped in growth of Indian agriculture but several germplasm lines of Indian origin have helped in saving crops in other countries. Some of the classical examples of use of germplasm of Indian origin are as:

- The entire rice crop of Indonesia was threatened some decades ago by a growth-stunting virus. A gene transferred from *Oryza nivara* from Odisha saved rice crop against the virus.
- It was single gene from India for downy mildew resistance that saved the muskmelon crop in the United States
- Another gene from Indian that provided American sorghum resistance to green bug insect had resulted in millions of dollars of annual benefit to American farmers
- Dr. William Saunders of Canada used wheat variety Hard Red Calcutta and released new series of wheat later called Marquis A and B which were early and resistant to rusts
- Recently in rice, *Sub1A* (from FR13A) and *PSTOL1* (from Kasalath) are being used globally to save rice from losses due to flooding and improving P use efficiency

Plant Quarantine

- Introduction of planting material, including transgenics from other countries carries risk of entry of the associated pests (fungi, bacteria, viruses, insects, nematodes and weeds etc.). Hence, all genetic resources acquired from foreign countries are tested using plant quarantine measures (legislative measures) to prevent the entry of exotic pests and to avoid their spread to the fields.
- ICAR-NBPGR has been empowered under the Plant Quarantine (Regulation of Import into India) Order 2003 of the Government of India to carry out quarantine checks on the germplasm being exchanged meant for research purposes, including transgenics. It undertakes quarantine processing of germplasm meant for export and issues the Phytosanitary Certificate for the material meant for export.
- The quarantine has resulted in the interception of several pests of high economic significance including (>70) those not yet reported from the country. Such interception signify the success of quarantine as otherwise these pests could have entered the country and played havoc with the plant biodiversity and Agriculture.
- So far 35,67,347 samples of various crops have been processed for quarantine clearance
- Infestation/ infection/ contamination detected in 1,96,324 samples of which 1,91,501 salvaged and 75 exotic pests intercepted.

Any inadvertent introduction of any pest not present in the country could lead to serious economic losses to farmers and the country. Estimated losses that could occur, if any of the diseases/weeds was introduced in the country are given in Table 2.

Table 2: Probable annual losses due to various exotic pests, if introduced into India

Crop	Pest	Yield loss in exporting country (%)	Probable annual loss in India (₹. in million)*
Wheat	*Monographella nivalis* (fungus)	3.0 to 52.4 (USSR)	1519.4
	Barley stripe mosaic virus	Up to 30.0 (USA)	
	Bromus secalinus (weed)	28.0 to 48.0 (USA)	
Soybean	*Bean pod mottle virus*	Up to 52.0 (USA)	261.9
	Peronospora manshurica (fungus)	Up to 80.0 (USA)	
Cotton	*Anthonomus grandis* (insect)	Up to 51.0 (USA)	221.4
Maize	*Maize chlorotic mottle virus*	90.0 (USA)	31.1
	High plains virus	Up to 100.0 (USA)	

*Losses based on the assumption that if only 0.1% yield loss occurs due to appearance of pest. The total yield and minimum support price have been taken for 2015-16, Source: http://eands.dacnet.nic.in/)

PGR Conservation

The Indian National Genebank (NGB) was established at ICAR-NBPGR to conserve the PGR for posterity in the form of seeds, vegetative propagules, *in vitro* cultures, budwoods, embryos/embryonic axes, genomic resources and pollen. The NGB has four kinds of facilities, namely, Seed Genebank (-18°C), Cryogenebank (-170°C to -196°C), *In vitro* Genebank (25°C), and Field Genebank, to cater to long-term as well as medium-term conservation.

The NGB with a capacity to conserve about one million germplasm in the form of seeds is currently conserving about 0.43 million accessions (Table 3) belonging to nearly 1,800 species. Over 12, 000 samples of seed, dormant buds, and pollen are cryopreserved (Table 4) and about 1,900 accessions are conserved in the *in vitro* genebank (Table 5). The NGB is supported by active partnership of other intuitions designated as the NAGS. The NAGS are responsible for maintaining, evaluating and distributing germplasm from their active collections to NGB and other user scientists.

Table 3: Germplasm conservation at ICAR-NBPGR Seedbank (as of March, 2019)

Crop/ Crop Group	Total Species	Present status of total acc. conserved
Cereals	124	164400
Millets	26	59268
Forages	195	7083
Pseudocereals	55	7585
Legumes	106	66350

Oilseeds	80	59161
Fibre crops	77	15704
Vegetables	210	26317
Fruits & Nuts	65	275
Medicinal & Aromatic plants & Narcotics	672	8064
Ornamental	120	657
Spices and Condiments	28	3135
Agroforestry	190	1646
Duplicate safety Samples (Lentil, Pigeonpea)	0	10235
Trial Material (Wheat, Barley)	0	10771
Total	**1948**	**440651**

Table 4: Status of germplasm in *In vitro* Genebank (as on March, 2019)

Crop group	Total accessions (no.)
Tropical fruits (banana)	430
Temperate and minor fruits (apple, apricot, blackberry, blueberry, pear, strawberry)	350
Tuber crops (sweet potato, taro, yam)	518
Bulbous and other crops (garlic, gladiolus)	171
Medicinal and aromatic plants	172
Spices and industrial crops (ginger, turmeric, pepper, cardamom, vanilla, hops, jojoba)	227
TOTAL	**1,868**

Table 5: Status of germplasm in Cryo genebank (as on March, 2019)

Categories	Total no of accessions*
Recalcitrant & Intermediate	**6,782**
Fruits & Nuts	3,520
Spices & Condiments	152
Plantation Crop	88
Agroforestry & forestry sp.	1,645
Industrial Plants (Jatropha, Pongamia, Salvadora, oil palm)	1,341
Medicinal & Aromatic Plants, Orchids	36
Orthodox#	**3,902**
Cereals	289
Millets and Forages	293

Pseudo-cereals	76
Grain Legumes	813
Oilseeds	668
Fibres Crops	68
Vegetables	581
Medicinal & Aromatic Plants	1,001
Narcotics & Dyes	35
Miscellaneous Sp.	78
Dormant Buds	**387**
Pollen Grains	**572**
Genomic Resources	**1,934**
Total	**13,577**

PGR Characterization and Evaluation

The utilization of PGR in crop improvement programs rests on identification of promising accessions. The collected or introduced germplasm is characterized and evaluated to assess its potential, by recording data on agronomic traits such as yield, quality, and tolerance to biotic and abiotic stresses. The germplasm is also evaluated for new traits using molecular tools to identify the genes to develop new varieties as per requirement of the farmers. Salient achievements are as:

- Approximately 10,000 accessions are characterized/ evaluated every year at ICAR-NBPGR and its regional stations. Till date, more than 2.35 lakhs accessions of different agri-horticultural crops have been characterized and evaluated and passport data is available.
- Core sets have been developed in four crops viz., okra, mungbean, sesame brinjal and wheat to facilitate the enhanced utilization of germplasm.
- Genetic diversity in large collection has been determined using morphological and DNA fingerprinting markers in crops like rice, mungbean, banana, cashew, mango, oilseeds, brassicas, tomato, sesame, cucumber and cotton.
- Mega programme on characterization and evaluation under the National Initiative for climate Resilient Agriculture (NICRA) executed in collaboration with SAUs for 21,822 accessions of wheat and 18,775 of chickpea.

DNA Fingerprinting and development of Genomic Resources

- About 2,300 varieties in more than 35 crops have been fingerprinted so far (Table 6). Also, the new varieties are being DNA fingerprinted to avoid any biopiracy by any unauthorized person or country.

- Established the National Genomic Resources Repository to collect, generate, conserve and distribute genomic resources for agricultural research in the country. The aim is to promote deposition, sharing and utilization of enormous amount of genomic resources generated in the country and elsewhere.
- All forms of genomic resources including clones, gene constructs, large DNA fragment libraries as well as genomic sequence information in soft copy form can be deposited in this repository.
- All depositions or requests are to be made along with material transfer agreements in order to protect the interest of the depositor and the sovereignty of the Nation over the genetic resources. The IP rights (if any) shall remain with the depositor.
- Newly identified genes (952) are also conserved in the form of DNA libraries, etc.

Table 6. Details of the crop varieties fingerprinted at ICAR-NBPGR

S. No.	Crop	No. of Varieties	S. No.	Crop	No. of Varieties
1	Rice	729	16	Safflower	26
2	Wheat	108	17	Saffron	13
3	Barley	54	18	Chickpea	77
4	Maize	140	19	Jute	31
5	Finger Millet	11	20	Oats	9
6	Sorghum	57	21	Pea	43
7	Pearl Millet	53	22	Lentil	25
8	Soybean	69	23	Mustard	42
9	Pigeonpea	49	24	Linseed	46
10	Mungbean	78	25	Cotton	116
11	Urdbean	76	26	Sunflower	7
12	Ricebean	4	27	Bittergourd	38
13	Mothbean	2	28	Mango	23
14	Cowpea	11	29	Cashew	105
15	Sesame	52	30	Tomato	30
Total: 2,195 (2015-17) DNA fingerprinting (Status) for protection of over 6440 released varieties of 52 species and native landraces to prevent unauthorized commercial exploitation.					

ICAR-NBPGR also has the mandate to carry out molecular profiling of varieties of agri-horticultural crops. This involves generation and utilization of molecular markers for molecular characterization of these crops. Although a lot of dominant multilocus marker systems such as RAPD (Random Amplified Polymorphic DNA), AFLP (Amplified Fragment Length Polymorphism) etc. have been used, the preferred markers for molecular characterization are SSR (Simple Sequence Repeat) and SNP (Single nucleotide Polymorphism) markers owing to their codominant nature. Whereas a lot of molecular

markers are available in crops like rice, wheat, maize, potato, cotton, soybean etc. there are others which despite being important contributors to the food basket are less worked at the genomics front.

Generation, validation and utilization of genomic resources is one of the major objective of ICAR-NBPGR. These resources are utilized for value addition to the plant germplasm resources harboured in the genebank and for generating molecular profiles varieties of agri-horticultural crops. The advent of next generation sequencing with improved chemistries and lower input costs have resulted high throughput data that can be mined for generating SSR and SNP markers.

- Genomic SSRs have been generated and validated at NBPGR in crops like okra, snake gourd and moth bean using NGS technologies in house. SSRs from transcriptome sequencing have been mined and validated in finger millet ,kodo millet, little millet and sponge gourd
- A novel gene targeted marker technique CBDP (CAAT Box-Derived Polymorphism) has been developed that can be used for various genotyping applications in plants. The technique exploits conserved CCAAT motif in the CAAT box region of promoter region of plant genes to generate markers. The concept has been validated in three different crops (Jute, cotton, linseed) representing five different species (*Corchorus capsularis, C. olitorius, Gossypium hirsutum, Gossypium orboratum*) and linseed (*Linum usitatissimum*).
- A draft genome assembly of 480 Mb of black pepper (*Piper nigrum*) genome has been generated at NBPGR under the ICAR funded Consortium Research Platform on Genomics. A large number of genomic resources in the form of genomic and genic SSRs have been generated.
- A rice core consisting of 701 accessions has been developed from 6,984 accessions of North-Eastern region of India. This core was further characterized with 50K SNP chip of Rice for development of mini-core. Cluster analysis based on 50K SNP markers grouped 192 accessions of core into seven clusters. This analysis shows that the core developed from NE rice collection is very diverse and has captured maximum diversity present (Figure 1).
- DNA Barcoding loci rbcL, matK, trnH-psbA and ITS region alone and/combination of two loci identified 21 genomic species in *Oryza* and were used for establishing correct genetic identity of mis-labeled species. Two combined loci DNA barcodes (rbcL + ITS) gave better species delineation and proper barcode gaps for species identification in genus Luffa.
- DNA profiling services were rendered to various public and private sector organizations. A total of 558varieties belonging to 33 crops have been profiled in the last four years itself.

- Discovery of non-Kranz C4 photosynthesis in two cell layers (cross- and tube-cells) of pericarp in developing wheat grains. Named it as "Bose anatomy" in honour of his earliest works on C4 in Hydrilla reported in 1924 when C3 itself was not known.
- Technology has been developed for Identification of SRAP Markers Linked to the Single Dominant Resistance Gene against Tomato Leaf Curl New Delhi Virus in *Luffa cylindrica* Roem: Two sequence-related amplified polymorphism (SRAP) markers closely-linked to the ToLCNDV-susceptible gene in the susceptible parent and in a susceptible bulk population; and two SRAP markers closely-linked to the resistance gene in the resistant parent and in a resistant bulk population were found. These can be used for large-scale screening of genotypes of *L. cylindrica* for resistance against ToLCNDV at the seedling stage, and to accelerate the breeding of high yielding, ToLCNDV resistant varieties and hybrids.
- Qualitative and quantitative PCR and real-time PCR assays have been developed/ validated for detection of more than 50 events of 14 GM crops (brinjal, cabbage, cauliflower, cotton, maize, mustard, oilseed rape, okra, papaya, potato, rice, soybean, tomato, wheat).
- Rapid and cost-efficient assays have been developed for screening of GM crops employing a) visual and Real-time Loop-mediated Isothermal Amplification (LAMP) for rapid on-site detection; b) GMO Screening Matrix as decision support system, and c) ready-to-use TaqMan® Real-time PCR based Multi-target system.
- GM-free Conservation of Germplasm in National Genebank: Bt cotton has been commercially cultivated in India since 2002 and other GM events of cotton, brinjal, okra and maize were under field trials, hence, to ensure GM-free conservation of germplasm in the National Genebank, the adventitious presence of transgenes was monitored in ex situ collection including cotton (200 accns.), brinjal (150 accns.), okra (50 accns,), maize (100 accns) using PCR/real-time PCR-based markers. None of the accessions screened so far showed adventitious presence of transgenes based on tests conducted.

Germplasm Utilization

The Bureau has supplied germplasm, collected indigenously or from exotic sources, to the breeders and other researchers in the country. The germplasm supplied by ICAR-NBPGR to various breeders have been used in varietal development. Several indigenously supplied germplasm accessions have helped to develop improved varieties in various national programmes. These include rice variety (Maruteru sannalu), sorghum variety (Parbhani Moti), red okra (Aruna), Chinese potato (Nidhi), coriander variety (Sudha), and yam variety (Indu) a few to name.

The NBPGR is involved in the release of about 100 varieties in the past in different agri-horticultural crops either through direct introduction or by selection from the introduced

germplasm and popularized several such introductions for commercial cultivation. Also many temperate fruits including kiwi, hops and several medicinal and aromatic plants like rose geranium are getting increasingly popular in Himalayan states, Uttarakhand and HP.

PGR Documentation

A PGR Portal has been hosted on NBPGR website, which is a gateway to information on plant genetic resources conserved. The Portal contains information on about 0.4 million accessions belonging to about 1800 species. The PGR documentation is done in various forms including printing of books, crop catalogues, inventories, research papers, popular articles, pamphlets etc. In addition, NBPGR has developed mobile apps Genebank and PGR map in PGR Informatics which can accessed through NBPGR web pages, genebank.nbpgr.ernet.in and http://pgrinformatics.nbpgr.ernet.in/pgrmap/

- Two mobile apps "Genebank" and "PGR Map" have been developed to enhance access to PGR information with an easy user interface. The apps have been hosted on Google Play and App Store.
- "Genebank App" provides a dashboard view of indigenous collections (state-wise), exotic collections (country-wise), addition of accessions to genebank, etc. The app also helps generate routine genebank reports. The app uses databases live on the backend and hence always gives updated information.
- "PGR Map App" offers three benefits: "*What's around me*" helps user to obtain quickly the accessions that have been collected and conserved in the genebank from a particular location in India where the user is located at the moment; *"Search the map"* helps user to list the accessions that have been collected and conserved in the genebank from any selected location in India; *"Search for species"* helps user to map the collection sites of a crop species.
- Establishment of geo-informatics portal in PGR: A study to link germplasm to changing climatic regimes was earlier carried out with the funding of the CGIAR Research Program on Climate Change, Agriculture and Food Security (CCAFS). A web interface named PGR CLiM was also developed to access information *(www.nbpgr.ernet.in:8080/climate).*

Germplasm Registration

Recognizing the importance of PGR with novel, unique, distinct and high heritability traits of value that could be used in crop improvement, and to facilitate flow of germplasm to users. ICAR-NBPGR plays a vital role in germplasm registration. More than 900 potentially valuable germplasm of over 120 species of various crops registered so far. To facilitate smooth registration process, a fully online system of filing registration applications, their scrutiny, review and communications at every stage has been developed

(http://www.nbpgr.ernet.in:8080/registration/). Details of the registered germplasm could accessed at http://www.nbpgr.ernet.in:8080/ircg/index.htm.

All India Coordinated Network Project Potential Crops

This network programme is located at ICAR-NBPGR, New Delhi and has 13 main centers in different parts of the country. The major functions are introduction, evaluation, conservation, and popularization of new potential and useful plant species for acclimatization to local condition. Grain amaranth, buckwheat, rice bean, jatropha and simarouba have been developed and popularized under this project.

Grain amaranth (*Amaranthus* spp.), normally grown in the hilly region, was tested for cultivation in the plains after the inception of the network in mid 1980s in Gujarat. The crop of grain amaranth acclimatized well and was found suitable for cultivation during rabi season. Three varieties, namely, GA-1, GA-2 and GA-3 were released for cultivation in north Gujarat. Likewise, germplasm for some other potential crops like quinoa (*Chenopodium quinoa),* faba beans (*Vicia faba*), etc. have been introduced and distributed to the farmers.

Human Resources Development

- NBPGR faculty conducts M.Sc and Ph.D. courses in PGR under the Post-graduate School of Indian Agriculture Research Institute (IARI), New Delhi.
- National and international training programmes conducted routinely on various aspects of PGR management.
- NBPGR is designated as the Centre of Excellence since 2006 by ICAR and Bioversity International to impart training on *in vitro* conservation and cryopreservation of PGR and more than seven trainings conducted. So far, more than 100 researchers from Bhutan, Colombia, Egypt, Fiji Islands, Ghana, Iran, Malaysia, Mauritius, Nepal, Nigeria, Papua New Guinea, Philippines, Saudi Arabia, South Africa, Sri Lanka, Taiwan, Thailand, The Czech Republic and Vietnam.

National and International Linkages

- Close collaborations with Bioversity International, ICARDA, IRRI, CIMMYT and other countries on genetic resources management and utilization
- Collaborations with all ICAR institute, state agricultural universities, CSIR institute, DBT, DST, DRDO etc. for germplasm trait-specific evaluation and utilization
- Memorandum of Understanding (MoU) was signed between the Indian Council of Agricultural Research (ICAR) and the Royal Botanic Gardens (RBG), Kew, UK to enhance capacities of both the institutions in research on conservation biology.

- ICAR-National Bureau of Plant Genetic Resources and Forest Research Institute, Dehradun had signed a MoU for the conservation of seed bearing trees species of forestry importance.
- MoU of ICAR-NBPGR with CPCRI, Kasaragod and NRC Orchids has been operationalized for cryo-conservation of coconut and orchids germplasm, respectively, at the National Cryogenebank, NBPGR.

ICAR-NBPGR for Safeguarding Nation's Future Food and Nutritional Security- Safety Duplicates: ICAR has taken a step forward in securing its crop genetic diversity by depositing 25 accessions of pigeonpea in the Svalbard Global Seed Vault. This was the first such deposit by India as safety duplicates in the global genebank which is jointly maintained and managed by Norway's Department of Agriculture and the Global Crop Diversity Trust under the ITPGRFA. A second deposit of 100 samples each of rice and sorghum was sent to Svalbard Global Seed Vault (SGSV) for safety duplication in 2016.

Initiatives and Developments

- **Gap Analysis and Biodiversity Mapping using GIS tools:** Mapping of collected diversity followed by gap analysis was done in some crops. In rice, more than 35, 000 accessions; in maize > 8, 000 accessions and in sesame over 2, 500 accessions collected from different states of the country were georeferenced.
- **Identification of useful gene sources in secondary and tertiary gene pool of Chickpea and Lentil**: Pre-breeding and genetic enhancement efforts in lentil and chickpea identified sources with specific traits. One representative set of global wild *Lens* accessions was developed by extracting 96 accessions using PowerCore approach.
- **Identification of climate analogues in crops for enhancing adaptive capacity to climate change**: Changing climatic regimes demand identification of specific prospective genotypes to be fed to the varietal development chain. ICAR-NBPGR linked attributes of the *ex situ* germplasm collections, i.e. agronomic descriptors and geographic origins with current and future environmental data. 84 accessions (pre-adapted to predicted changes) belonging to pearl millet, chickpea, pigeon pea and sorghum crops were identified.
- **CRP on Agro-biodiversity** to undertake characterization of entire genebank collections and evaluation in select crops. The Platform has specific sub-projects addressing genetic resources management issues related to plants, animals, fish, microbes and insects. The respective Bureaus coordinate these management functions involving other stakeholders in their area domain.
- **CRP on Genomics** was initiated with a focus on generating genomic resources for value addition to PGR for genetic improvement of crops. The establishment of a

genomics platform would provide a state of the art infrastructure and expertise for carrying out genomics work at all NARS/ICAR institutes including five Bureaux.

- **Technologies transferred**
  - Application of DNA-based markers to differentiate citrus root stocks.
  - PCR based detection assays and protocols for ten genetically modified (GM) crops.
  - Five technologies related to DNA-based GMO screening, viz., Hexaplex PCR targeting six marker genes; Duplex TaqMan® Real-time PCR targeting P-35S and T-nos; Visual Loop-mediated Isothermal Amplification (LAMP) targeting eight transgenic elements; Real-time LAMP targeting eight transgenic elements; TaqMan® Real-time PCR based multitarget system targeting 47 targets.

Future Thrusts for ICAR-NBPGR

- Geo-referencing of all the indigenous germplasm and superimpose it with soil and climate maps.
- Targeted germplasm collection based on gap analysis.
- Evaluation of germplasm for target traits including biotic, abiotic stress, nutritional and processing traits.
- Strengthening of post-entry quarantine.
- Geo-referencing of perennial crop (fruit and forest tree) germplasm.
- Establishment of Field Genebank for semi-arid fruits at Issapur farm of ICAR-NBPGR.
- Establishment of clonal repository of temperate fruits.
- Generation of robust cores based on high throughput genomics resources and phenotypic traits in all the major crops.

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2

Some Important Plant Resources from Western Ghats

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Western Ghats is one of the 36 hotspots and one of the eight hottest hot-spots of the globe. It occupies 5% of Indian's total area but possesses 25-27% of Indian flowering plants. Presently round about 7400 species of angiosperms are recorded from Western Ghats of which 5588 are indigenous. Endemic angiosperms in Western Ghats are about 2255 of which about 1275 are exclusively known from the region. About 20-25 species of flowering plants are being described from Western Ghats every year. Western Ghats play important role in hydrological cycle in the peninsular India and most of the perennial rivers originate in Western Ghats. In other words, Western Ghats is backbone of peninsular India. The region is blessed with high biodiversity uniquely adapted to Western Ghats environment. It forms important region of animal and plant sources. There are several plant resources in Western Ghats which are yet to be explored and utilized. Several plant sources of food, fodder, timber, gum, resins, medicine, ornamental and commercial value are available across the region.

Plants such as *Abutilon ranadei, Arisaema species, Bigonia tricocarpa, Camptorrhiza indica, Ceropegia* species, *Crinum* species, *Delphinum malabaricum, Dipcadi concanense Frerea indica, Habenaria crinifera, Impatiens* species, *Paracaryum malabaricum* and number of others have ornamental value and could be domesticated and introduced in horticultural trade. Some of the rare shrubs which have large beautiful flowers of ornamental value include *Abutilon ranadei, Argyeia cuneata, Barleria* species, *Beaumontia jerdoniana, Calacanthes grandiflorus, Carvia callosa, Decaschistia trilobata, Ochna obtusata, Nilgirianthes* species, *Pleocaulis ritchei, Supushpa serobiculata* which deserve place in garden. Many of the herbaceous plants have flowers of ornamental value and could be introduced in gardens as seasonal. Important herbaceous plants known for beauty of their flowers include species of *Alysicarpus, Arisaema, Bigonia, Chlorophytum, Crinum, Curcuma, Cyanotis, Delphinium, Dendrobium, Habenaria, Impatiens, Murdania, Smithia* and others- *Adelocaryum coelestinum, Aerides maculosum, Paracaryum malabaricum, Pinda concanensis* and many others.

Plant species which could be introduced into gardens as evergreen trees include *Arenga wightii, Calophyllum apetalum, Cinnamomum goaense, Dimorphocalyx lawianus, Diospyros paniculata, Elaeocarpus munroii, Euonymus indicus, Garcinia talbotii, Gordonia obtusa, Knemma attenuate, Maba nigrescens, Memecylon talbotianums, Microtropis* species, *Meiogyne pannosa, Myristica malabarica*. *Phaeanthus fragrans, Pittosporum dasycaulon,*

Phoenix robusta, *Sageraea laurifolia*, *Syzygium laetum*, *S. stocksii* and numbers of others. Plants such as *Ancistrocladus heyneanus*, *Asparagus* species, *Curcuma* species, *Delphinium malabaricum*, *Heracleum* species, *Holigarna* species, *Hydnocarpus pentandra*, *Moullava spicata*, *Zingiber* species and many others are of medicinal value and so far the plant materials of medicinal plants are mainly exploited from wild sources. There is need to cultivate at least important, rare medicinal plants. It is a need of present time for continuous supply of drug plants.

The seeds and corms of *Camptorrhiza indica*, *Gloriosa superba* and *Iphigenia* species contain colchicine. The members of these genera could be a commercial source of colchicine (Kapadia *et al*., 1972, Ansari and Rao, 1973, 1978). Of them, *Iphigenia stellata* is one of the most promising sources of colchicine. There is need to find out cultivation practices for commercial exploitation of colchicine from these plants. The bulbs of *Drimia* species contain number of alkaloids used in medicine especially in heart troubles. In India, there are about 9 species of *Drimia* of which *D. coromandelliana* (tetraploid) from Kolhapur region contains highest glycosides and is promising source of glycosides. Cultivation and improvement of strains through genetic manipulation is worth trying in commercial productions of glycosides. Beside this, different *Drimia* species have number of other uses.

Plant species of food value in the form of tubers, corms and bulbs for wild life include species of *Amorphophallus*, *Brachystelma*, *Ceropegia*, *Dipcadi*, *Habenaria* and *Plantanthera susannae* etc. Rarity of some species of *Ceropegia*, *Habenaria*, *Plantanthera* is attributed to destruction of tubers by wild herbivorous animals. Species of *Amorphophallus*, *Brachystelma*, *Ceropegia*, *Chlorophytum*, *Cucumis setosus*, *Flemingia neilgheriensis*, *Merremia rhyncorhiza* have tuberous edible root stock or fruits of food value. Some of them could be introduced as tuberous crops. There is need of research on wild sources of food and their domestication. Number of rare plants from Western Ghats are of botanical interest. Although uses of most of the rare plant species such as *Bhidea bursiana*, *Dalzellia zeylanica*, *Danthonidium gammiei*, *Eriocaulon* species, *Frerea indica*, *Glyphochloa species*, *Griffithella hookeriana*, *Hydrobryopsis sessilis*, *Hyphaene dichotoma*, *Indopoa paupercula*, *Oianthes* species, *Podostemon subulatus*, *Polypleurum dichotomum*, *P. stylosum*, *Pseudodichanthium serrafalcoides*, *Seshagiria sahyadrica*, *Trithuria konkanensis*, *Triplopogon*, *Trilobachne cookei*, *Utricularia* species, *Wiesneria triandra* etc. are presently not known, but they have great importance in understanding biogeography and evolution of plants.

In addition to potential values of plants from Western Ghats, some of the wild plants form important genetic pool to improve our timber, fiber, food and fruit plants through hybridization and selection of desired genes for disease resistance, food value and yield. Important wild germplasm include species of *Asparagus*, *Atylosia*, *Crinum*, *Curcuma*, *Garcinia*, *Zinziber*, members of Poaceae, Fabaceae and Vitaceae, *Cucumis setosus*, *Vigna khandalensis* and number of others. Aquatic plants such as *Aponogeton bruggenii*, *A. satarensis*, *Cryptocoryne cognata*, *Eriocaulon dalzellii*, *E. breviscapum*, *E. setaceum*,

Eusteralis species, *Nymphoides aurantiacea*, *Rotala ritchei* and *Utricularia* species have potential value as aquatic plants for aquaria. They have good growth forms which enhance the beauty of aquarium.

None of the plant is useless and it is necessary to conserve maximum biodiversity. Very little is known about these plants and their potential values, however, many of the vanishing plants of Western Ghats have either ornamental, medicinal, botanical or some value. In spite of ornamental values of our indigenous plants, it is surprising to see that even 5% have not found place in our gardens. Over 95 % plant species grown in gardens are exotics. This clearly indicates that no serious efforts were and are made to domesticate, increase the ornamental value and to use these indigenous plants in garden. Indo-American Hybrid seed farm has evolved over 100 varieties of *Lantana* alone and shown the way how wild plants can be utilized. In recent years some of the wild plants are introduced in the gardens. Some of the rare endemic plants which have found place in garden are *Ceropegia juncea* and *Frerea indica* of which latter species is critically endangered. There is a need to survey the rare wild plants through extensive field studies, know their present status and potentials as commercial, medicinal, ornamental plants or any other use. In addition to *in-situ* and *ex-situ* conservation, there is need to propagate, cultivate, domesticate and introduce these rare plants in horticultural trade. Some of the wild plants can be introduced as food crops through domestication. Some of them are of industrial importance. Once the plant species is made useful to man, people will cultivate them to reap the benefits from it. This will help not only in conservation of rare, endemic plants but also their utilization in sustainable development of the country. There is a need to know autecology of at least critically endangered plant species of the country.

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3

Exploration and Germplasm Collection: Status, Priorities and Future Thrust

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Plant genetic resources (PGR) constitute our invaluable assets to meet the growing demands to increase crop production and productivity. Genetic resources are most important components of present and future crop breeding programmes. Augmentation of germplasm is the first and the foremost activity in the PGR management system, therefore meticulous planning and exploration following scientific principles governing the diversity distribution is crucial. There are reports of sporadic collections of indigenous crop germplasm during the earlier part of 20th century, for instance, wheat from north-western plains (Howard and Howard, 1910), jute (Burkill and Finlow, 1907) and few pulses. Significant progress has been made subsequently, in collecting and conservation of indigenous and exotic plant genetic resources (PGR) by several countries like, USA, China, India, United Kingdom, Germany, Japan and international centers of CGIAR (mainly CYMMET, IRRI, ICARDA, ICRISAT, CIAT and IITA).

Indian gene centre belongs to one amongst the 12 mega gene centres of the world and endowed with rich agro-biodiversity. It is also one of the Vavilovian centres of origin and the diversity of crop plants (Table 1). Among the 34 biodiversity hot-spots of the world, India has four, *viz.*, Himalayas, Western Ghats, Indo-Burma and Sundaland (Nicobar Islands). The country is estimated to have over 48,000 plant species representing 12.5 per cent of the total world diversity (Singh *et al.,* 2000).

India has over 18,000 angiosperms (7.2% of the world total) with 29% endemic species. A total of 166 out of 2,489 plant species distributed in 12 regions of diversity of cultivated plants (Zeven and de Wet, 1982) including 25 major and minor crops have originated and / or developed diversity here; over 480 species of cultivated plants occur here (Nayar *et al.,* 2003); over 9,500 species of ethno-botanical importance have also been reported, of which 3,900 are used for food. Crops in which rich diversity occurs in the country include rice, wheat, maize, barley, pigeonpea, chickpea, minor millets, mung bean, urdbean, horsegram, moth bean, ricebean, cluster bean, sesame, forage grasses, okra, eggplant, cucumber, melons, citrus, banana and plantains, jackfruit, mango, tamarind, jamun, jute, cotton, ginger, turmeric, pepper, cinnamon and cardamom. Varied topography, diverse ecosystems and rich agricultural heritage coupled with ethnic diversity have contributed enormous richness of

plant genetic resources (PGR) in the country, including germplasm having potential for combating with abiotic stresses like salinity, drought, cold and heat. India has 40 identified agro-biodiversity spots in Western Himalaya, Eastern Himalaya, Eastern Ghats, Aravalli, Eastern region of India and Vindhyan ranges (Nayar, 1996).

Presently, over 500 species of both indigenous and exotic cultivated plants are widely grown in different agro-ecological regions of the country. The crop wild relatives have played a significant role in crop improvement. Recently, about 4,157 utilizations of CWR in crop improvement, spanning 127 crops have been reported. In India, 833 taxa belonging to 741 species, including wild/weedy form(s) or populations of 145 crop species have been shortlisted as CWR in India and among these 238 species have been prioritized for exploration and collection (Pradheep *et al.*, 2014).

Table 1. Major crop species of Indian origin

Crop group	Crop (botanical name)
Cereals and millets	Rice (*Oryza sativa*), little millet (*Panicum sumatrense*), kodo millet (*Paspalum scrobiculatum*)
Grain legumes	Black gram (*Vigna mungo*), moth bean (*V. aconitifolia*), pigeonpea (*Cajanus cajan*), horse gram/kulthi (*Macrotyloma uniflorum*), velvet bean (*Mucuna utilis*)
Fruits	Mango (*Mangifera indica*), banana (*Musa* spp.) jamun (*Syzygium cumini*), jackfruit (*Artocarpus heterophyllus*), *Citrus* group, lime and others, karonda (*Carissa congesta*), khirni (*Manilkara hexandra*), phalsa (*Grewia asiatica*), bael (*Aegle marmelos*), wood apple (*Feronia limonia*), kokam (*Garcinia indica*)
Vegetables	Eggplant (*Solanum melongena*), ridged gourd and smooth gourd (*Luffa* spp.) round gourd/tinda (*Praecitrullus fistulosus*), pointed gourd/parval (*Trichosanthes dioica*), taro/arbi (*Colocasia esculenta*), yam (*Dioscorea* spp.), jimikand (*Amorphophallus campanulatus*), kundri (*Coccinia indica*), cucumber (*Cucumis sativus*), rat tailed radish/mungra (*Raphanus caudatus*)
Oilseeds	Rai, sarson and toria types (*Brassica* spp.)
Fibres	Jute (*Corchorus capsularis*), cotton (*Gossypium arboreum*), sunnhemp (*Crotalaria juncea*)
Medicinal and aromatic plants	*Rauvolfia serpentina, Saussurea lappa,* Indian belladonna (*Atropa acuminata*), Indian barberry (*Berberis aristata*), Guggul (*Commiphora wightii*)
Spices and condiments	Turmeric (*Curcuma domestica*), ginger (*Zingiber officinale*), cardamom (*Elettaria cardamomum*), Bengal/large cardamom (*Amomum aromaticum*), long pepper (*Piper longum*), black pepper (*Piper nigrum*), betle leaf (*Piper betel*), cinnamon (*Cinnamomum* spp.)
Other crops	Sugarcane (*Saccharum officinarum*), bamboos (*Bambusa arundinacea*), *Dendrocalamus hamiltonii*, *Sinocalamus giganteus*), *Sesbania sesban*, tea (*Camellia sinensis*)

Status of germplasm collection in India

The collecting of plant genetic resources (PGR) primarily aims at tapping available variability in different agri-horticultural crops, their wild relatives and related species. The germplasm so collected reveals the nature and extent of variability in different species, within species, cultigens, etc. as also their agro-ecological/phyto-geographical distribution. It is obvious thus that much PGRs had been explored, collected and studied in the later part of the nineteenth century. The knowledge so gained formed the basis of further studies and diverse genetic resources got assembled for use in crop improvement programmes (Mehra and Arora, 1982; Paroda and Arora, 1986). The search for new genes got initiated through the foresight of late Dr. B.P. Pal in 1940s and plant exploration and germplasm collection activity got impetus (Pal, 1937).

In India, systematic plant exploration and germplasm collection work was initiated with the establishment of central agency in 1946 in the Division of Botany, Imperial Agricultural Research Institute, New Delhi. Late Dr. Harbhajan Singh, an eminent and pioneer plant explorer initiated germplasm collection and significantly contributed in the field of plant genetic resources. This activity has been more systematized after creation of National Bureau of Plant Genetic Resources in 1976. This activity has been more systematized after the creation of the National Bureau of Plant Genetic Resources (NBPGR), New Delhi in 1976. Prior to this, between 1946 to July 1976, a total of 31,235 germplasm accessions of cereals, millets, legumes, oilseeds, vegetables, fiber yielding and other economic plants including wild relatives were collected (Table 2).

Table 2: Indigenous germplasm collections made prior to the creation of NBPGR

Period	Collections	Crop groups
1946-1950	615	Cereals, millets, legumes, oilseeds, vegetables, fibre yielding and other economic plants including wild relatives.
1951-1955	3,363	
1956-1960	4,245	
1961-1965	4,470	
1966-1970	1,902	
1971-1975	10,737	
1976 (upto July)	5,603	
Total	**31,235**	

This activity received greater impetus from 1985. Up to 1998, major emphasis was on crop-specific collaborative explorations (Gautam *et al.,* 1998). Majority of exploration missions were multi-crop/region specific surveys, while after 1976 till 1984 both multi-crop/region specific and crop- specific surveys were made. Later, for the one and half decade, collaborative explorations with crop based institutes received greater impetus. After CBD in 1993, germplasm was considered as sovereign property of the nation. The urgency was felt and NBPGR/ICAR rose to the occasion. With the help of World Bank aided project National

Agricultural Technology Project on Plant Biodiversity, this activity has taken a major boost from September 1999. It was a unique effort at national level, wherein more than 130 organizations representing all crop based Institutes, SAUs, other Governmental and Non-Governmental Organizations (NGOs) worked in collaboration for national cause of collecting and conserving agro-biodiversity. Several Indo-International programs on plant exploration and collection of specific crops and groups of crops were undertaken by IARI (the erstwhile Plant Introduction Division) and NBPGR in collaboration with certain international organizations or with financial support.

Since inception of the NBPGR, significant work has been done on exploration and collection of germplasm of different agri-horticultural crops and their wild relatives from diversity rich areas in country. From August 1976 to May 2019, 2740 explorations have been undertaken in which a large number of germplasm collections (2,75,692 including 207,760 cultivated and 36,697 wild types) of economically important agri-horticultural crops and their wild relatives have been collected from different agro-ecological zones/habitats involving more than 130 organizations including crop-based institutes, state agriculture universities (SAUs) (Table 2). The collections of different crops were categorized in to 13 crop groups viz. cereals, pseudo-cereals, millets & minor millets, pulses or grain legumes, vegetables, oil seeds, fruits, fibre and allied crops, fodder crops and grasses, medicinal and aromatic plants, spices and condiments, agro-forestry/ tree spp., sugarcane and other crops. Collection status of each crop group is presented in table 2. Highest collections have been made in cereals followed by vegetables and pulses. Collection status, gaps and priority areas of crops and their relatives are presented in paper under these crop groups.

Table 3: Germplasm of crop-groups collected by ICAR-NBPGR

Sl.No.	Crop-group	Accessions (1946- 2019)
1.	Cereals	60577
2.	Pseudocereals	7310
3.	Millets & minor millets	22521
4.	Vegetables	53983
5.	Pulses (grain legumes)	41151
6.	Oil seeds	25228
7.	Fruits	13820
8.	Fibre and allied crops	5559
9.	Fodder crops and grasses	2116
10.	M & AP, spices & condiments	27873
11.	Agroforesty/ tree spp.	2094
12.	Sugarcane	1232
13.	Other economic crops/plants	12228
Total accessions		**275692**

Gap analysis of augmented diversity: a new initiative

The term 'Gap analysis' refers to a systematic method of analyzing the degree of conservation of taxa, in order to identify those locations, taxa, and particular traits (adaptations) un- or under- secured in conservation systems (Maxted *et al.*, 2008). Technological advances in both software and hardware, together with the increased availability and accessibility of geographical, environmental and biodiversity data, have led to increased application of GIS (Geographical Information System) analysis for collecting, conservation and use of PGR in the last two decades. NBPGR has initiated gap analysis work of augmented indigenous germplasm accessions (1,30,984) of seven crops viz. rice (1,00,425), wheat (8,013), maize (8,375), pigeonpea (2,967), finger millet (3,898), fox-tail millet (5,891) and tomato (1,415) assembled from different states of the country (Semwal et al., 2017, Panwar et al., 2015, Pandey et al., 2014, Semwal et al., 2013). However, remaining precious germplasm (>3,06,911 accessions) of cultivated, underutilized crops and crop wild relatives (1,746 species) of the country need to be geo-referenced and gaps to be identified on priority basis. Gap analysis study is generally conducted to identify the 'gaps' in collections vs conserved diversity (Margules, 1989). The analysis is also used to assess the genetic and geographical diversity of crops and their wild relatives, as it allows to detect incomplete germplasm collections as well as to decide which species should be collected and in which locality these collections are to be focused.

Gap analysis can be categorized in to four steps: (1) to identify and classify biodiversity, (2) to locate areas recognized for biodiversity, (3) to identify biodiversity that is under represented in those known areas, and (4) to set priorities for new collection programme through ground truthing. GIS tools have been used to find out the gaps in existing collections and to identify areas for future collections, as well as to locate germplasm collection sites for potential/trait-specific crops, for the purpose (Hijmans *et al.* 2001).

Priorities and future thrust

Ex-situ Conservation of Plant Genetic Resources

For present and future use, diversity useful for mankind needs to be conserved using *ex-situ*. Focused collection of less collected/ unrepresentative germplasm of indigenous crop taxa belonging to prioritized crops namely vegetables and their wild relatives, millets & minor millets and their wild relatives; pulses/legumes and their wild relatives; oilseeds and their wild relatives; fruits of Indian origin, underutilized crops and cereals after gap analysis from targeted areas viz. un-explored/under-explored areas, diversity rich pockets, remote, inaccessible and tribal dominated areas, etc. for their ex-situ conservation by involving various stakeholders, ICAR crop-based institutes, SAUs, KVKs, NGOs and state government departments.

Mapping, documentation and cataloguing of genetic resources

India is richly endowed with plant wealth of cultivated crops and species of economic importance. However, the diversity available at species and genetic level, their distribution, habitat and niche areas of crop wild relatives (CWR), crops/ landraces, minor and potential fruits, medicinal and aromatic plants have not been systematically documented and mapped at fine grid level. Hence, proper inventorization and documentation of these resources can lead to identification of gaps in exploration, quantifying the genetic erosion component and device strategies to rehabilitate the lost diversity by recouping from gene banks. Mapping and documentation of plant diversity will be helpful in identification of vulnerable species and areas, study trends in diversity and predict impact of climate change. The database would be of great use in planning and use of natural resources and future studies. In this regard, PGR database, herbariums and secondary information would be used to document and map the diversity of prioritized crops/species of economic importance. Real time Remote Sensing data, GIS and ground truthing needs to be done involving Indian Institute of Remote Sensing (IISR) and Indian Space Research Organization (ISRO). Data pertaining to unique traits/characters, areas of occurrence, richness and distribution, *in-situ* sites, habitat dynamics, traditional knowledge/ cultural value of plant genetic resources should be documented. This is a gigantic task require involvement of all stakeholders under NARS (National Agricultural Research System), BSI, CSIR and others in central/state universities including experts in GIS, software development and data management to document and map the diversity in cultivated and economically important species of plants.

In-situ on-farm management of native agro-ecosystems

The crop wild relatives (CWR) have played a significant role in crop improvement, global food security in the wake of climate change, and require immediate conservation measures before these are lost. In India, 833 taxa belonging to 741 species, including wild/weedy form(s) of 145 crop species have been shortlisted. Of these, so far only 15% of CWR species diversity could be conserved in National Genebank (NGB). Besides, there are several wild species including minor fruits like kokum (*Garcinia indica*), jackfruit (*Artocarpus heterophyllus*), sahajan (*Moringa oleifera*), jamun (*Syzygium cumini*), bael (*Aegel marmelos*), ber (*Ziziphus mauritiana*), phalsa (*Grewia asiatica*), khajoor (*Phoenix dactylifera*), kronda (Carissa *carandas*), khirni (*Manilkara hexandra*), chironjee (*Buchananai lanzan*), custard apple (*Annona squamosa*), figs (*Ficus* spp.), mulberry (*Morus alba*), berries, *Rubus* spp., *Ribes* spp., *Prunus* spp., etc. are rich in nutrients, antioxidants and source of livelihoods for remote villagers in general and for tribal's and forest dwelling communities in particular.

Medicinal and aromatic plants are also rich in bio-molecules and widely used in Indian system of medicines, can boost the economy of India. Their *in-situ* conservation is very essential due to over exploitation and loss of species and genetic diversity. Majority of the abovementioned species are habitat/niche specific, growing in farms, forests and degraded community land, which are under severe pressure of habitat loss due to several reasons. It is

very essential to conserve these resources in their natural habitat to ensure adaptation and evolutionary process in changing climate, nutritional security and posterity. There is need to identify their habitat/niche specific sites for declaring them as *in-situ* conservation sites/ Biodiversity Heritage Sites. Status of species, population and genetic diversity at each site needs to be assessed taking help of remote sensing techniques to locate and identify the potential sites to study current status and temporal changes. Besides *in situ/ ex-situ* conservation, documentation of associated traditional knowledge is equally important as several species are being exploited by tribal people residing in remote/forest areas.

The representation of CWR in seed banks, in general, is inadequate and often collected sporadically (rather than systematic collection). The second *State of the World Report on Plant Genetic Resources for Food and Agriculture* reports about 10% share of wild species in the global germplasm holdings, in which CWR constitute about 2-6% (Maxted and Kell, 2009). Major impetus for CWR collection at national level was made during National Agricultural Technology Project on Sustainable Management of Plant Biodiversity (1999-2005) operational at NBPGR and afterwards, about 27 per cent of accessions collected every year (after 1999) belongs to wild species. This has resulted in about 12% share of wild species of PGR importance to the total collection in NGB of NBPGR, New Delhi. CWR occupy about 2.76% of the total collections in this genebank.

Mainstreaming agricultural biodiversity conservation and utilization

Agricultural biodiversity is the foundation of agriculture. It is the source of genetic material that is vital to future generations. The use of agricultural biodiversity can help make agricultural ecosystems more resilient and productive; and can contribute to better nutrition, productivity and livelihoods. However, there is significant loss in species, population and habitat of agrobiodiversity due to several reasons. It is a well known fact that most of the improved varieties have lower nutritional value than traditional landraces produced organically and generally failed under stresses (biotic and abiotic). Importance of on-farm management and sustainable use of Plant Genetic Resources for Food and Agriculture (PGR-FA) are recognized at the national and international level, and the key policy developments. *In-situ* on-farm management of farmers' varieties on smallholding/marginal farms provides a valuable option for conserving crop diversity for sustainable utilization. More importantly, it helps sustain evolutionary systems that are responsible for the generation of genetic variability. After achieving food security, the national focus would be on nutritional security and on climate resilient farming systems. For these, the ICAR-NBPGR is a key institution in the country that can make available the nutritionally rich widely adapted landraces and farmers varieties of crops for all types of growing conditions in the country. This endeavour will enhance capacity of farming and dependent communities by strengthening local seed supply systems and establishment of community seed banks (CSBs).

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4
Germplasm Collecting: Logistics and Tactics

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Exploration and germplasm collecting is a fascinating field of study that has attracted the attention of many adventurists, naturalists, travellers and plant hunters since time immemorial. Its main purpose is either gathering samples representative of the genetic variability of a particular species or looking for variability for a specific trait (e.g. tolerance to drought, salinity). Hence, depending on the needs and objectives, exploration missions are executed for collecting maximum variability from a given area/ region. The objective of germplasm collecting may be achieved if maximum genetic diversity is collected, properly documented and follow up research is undertaken on the collections and its results are made available to the scientific community (Guarino *et al.*, 1995). The systematic collecting and augmentation of diversity in crops started in the early twentieth century, initially in wheat and subsequently followed in other crops. Since the establishment of Convention on Biological Diversity (CBD) in 1993, plant genetic resources have become the "sovereign property" of the country in which they are found. This new interpretation initiated new concepts and requirements that shape the future of plant exploration. Under these new regulations, plant material can no longer be removed arbitrarily without due consideration of farmers, land owners, or sovereign territories. Due to changes in the international scenario in the recent years, for example Agenda 21, the Convention on Biological Diversity (CBD) and Global Plan of Action (GPA), exploration for monitoring and PGR collecting are expected to increase.

Reasons for germplasm collecting

The main reasons for collecting germplasm in given gene pool are that:

- it is in danger of extinction or even erosion particularly the wild species;
- a clear need exists for it, as expressed by users both at national and international levels;
- the diversity;
- it represents is missing from or insufficiently represented in existing *ex-situ* germplasm collection;
- rescue collecting-if genetic diversity is imminently threatened in an area and *in-situ* conservation methods are not feasible, germplasm collecting may be warranted;
- the loss of genetic diversity causing genetic erosion;

- the loss of genetic diversity due to over exploration; collecting for immediate use - Local communities especially engaged in traditional medicine are continuously collecting germplasm for immediate use;
- the gap filling for future use: the material not considered particularly useful today may turn out to be vital tomorrow;
- to address issues of intellectual property rights and Convention on Biological Diversity (CBD).

Logistics and tactics of germplasm collecting

Germplasm collecting tactics, logistics, preparations and procedures are discussed by several workers (Harlan 1975; Hawkes 1980; Arora 1981; Astley, 1991; NBPGR, 2014). However, some of the important tactics, logistics and steps are necessary to follow by an explorer are as below:

- Planning,
- Gathering required equipment and preparation,
- Making contacts with local research organizations,
- Meeting with local researchers and other officials including NGO's,
- Collecting the germplasm and herbarium specimens,
- Sorting out of collected samples,
- Reporting to the Headquarters,
- Preparation and publication of collection reports, and
- Delivering/distributing collected samples.

Before setting out for explorations in the target area, appropriate tactics and strategies and logistics are considered necessary for the germplasm collection as mentioned below:

Planning of collecting programme: To undertake any exploration and germplasm collecting programmes, planning in advance is essential for finalize the mission. But the present day technological advances enable explorers to sit at home or in the office, connect with the Internet, and, through the use of geological positioning devices (GPS), plan a collecting route that most likely will contain targeted species.

Area and route of exploration: For any successful exploration it is essential to have adequate knowledge of route to be followed. For this the purpose detailed route maps (state and district) should be procured for reference.

Time of collecting: Collecting is done when the crop is physiologically mature and is almost ready for harvest. The collectors should also collect samples of other crops maturing at the

same time. In case of collecting wild species missions are executed rather earlier (depending on crop/ species) or before their maturity.

Exploration items and equipments: A comprehensive list of exploration items and equipments is given below.

List of items and equipment for collecting

Survey/ collecting items	Global Positioning System (GPS), digital camera with additional memory card, binocular, magnifying glasses, handheld microscope, digital Vernier calliper and portable balance, Haversack/ kitbag, seed envelopes, cloth bags, polythene bags, aluminium & tag labels, drying sheets, old newspapers, plant press, moss, rubber bands, packing tape, *sutli* (thick and thin), secateurs, scissors, knife, digger, torch light, measuring tape, passport data book, field note book, pencil, ballpoint pen and permanent marker.
Reference material	Regional/ national flora, digital herbarium, lap-top and accessories, list of local names of plants, road-map, vegetation/climate map, list of rest-houses/ lodges, hotels, resting/ stay places and list of local contacts.
First aid-box	Anti-malaria pills, anti-allergen tablets, pain killers, anti- amoebic and anti-diarrhoeal tablets, mosquito repellent, antifungal/ antibacterial/ antiseptic creams or lotions, cotton-packs, band-aid, dettol, dressing gauze, water-purifying tablets, etc.

Collecting strategies and sampling method

Application of correct sampling method ensures that the genetic diversity of crop/species represented in the collected sample. For sampling the germplasm, region/site, inaccessible areas, valleys, isolated hills, villages at the edge of deserts, forests, mountains and isolated coastal belts which may hold rich genetic diversity, potential/ trait-specific germplasm and crop wild relatives should be approached for collecting. For cultivated species, farmers' field, backyard/ kitchen garden, threshing yard, farm store, local village market, etc. are ideal sites. Conducting explorations in unexplored areas, coarse grid surveys can be carried out to capture the overall variability, while fine grid survey is carried out to build-up collections for specific trait(s) known to exist in identified pockets in previously explored areas. The frequency of sampling (number of samples per site) should be decided based on on-the-spot observations on the variability available.

While collecting the seed, the explorer should keep in mind the required quantity of material to be sampled for long term conservation (2000 and 4000 seeds for self and cross pollinated crops respectively) besides meeting the requirement of characterization, evaluation and related studies. In general, random sampling should be followed to obtain a representative and adequate sample (Marshall and Brown, 1975). The random sample can be enriched with

biased or rare phenotypic variants. In a situation when wild population/crop wild relatives with few individuals occur, one should better collect from all the plants so as to make the representative sample from that site. Material with dubious identity or unidentified material, vernacular name should be collected along with herbarium specimen and photographs for authentication. The herbarium specimens can be collected as per procedures/guidelines (Jain and Rao, 1977; Pandey *et al.*, 2013)

Passport data recording: Information in the passport data needs to be recorded in the collecting site (Annexure I). Information on genetic erosion should also be gathered particularly on the depletion of landraces cultivated over the time and the reasons for their loss in general. Indigenous traditional knowledge is also to be asked and recorded. Meaning of the name of landraces and their properties should also be recorded. Observations on the distribution pattern and frequency status of crop wild relatives, rare, endangered and threatened species of PGR importance should be recorded for their sustainable management. Ethnobotanical observations and new uses of plants, especially those collected from tribal dominated tracts, are currently recorded as a database which would be available for reference in future collections.

Post collection handling: Post collection handling is also one of the most important activities to follow. The cleaning of seed should be done preferably on the same day or immediately after completing the expedition and process for their drying. The seeds with short longevity should be processed at the earliest and care should be taken during threshing/ cleaning to avoid damage. The observations on variability parameters should be recorded along with photographs for documentation and publication. One set of the properly labelled material along with passport data should be deposited for accessioning, conservation and another one to be accompanied by the collaborator for initial seed increase, maintenance, characterization and evaluation. The vegetatively propagated material should be sent for establishment/ maintenance in field genebank or at suitable site. The material for *in vitro* and cryo-genebank should be handed over for cryobanking.

Report on collecting mission: After completing the exploration and germplasm collecting mission, a comprehensive report should be written to fulfill the mission's objectives. This helps in determining if follow-up collection(s) would be needed and provides information to users about availability of the germplasm.

Collection challenges into the future

Once the expedition reaches a collection location, difficulties may exist in finding the desired species. Intrusion of human development or in situ ecological changes may reduce rare species with limited distribution or diversity within populations of more widely distributed species. Sometimes fruits and seeds are found only at the top of tall trees. In the past, younger individuals were contracted to climb trees to obtain seeds for collections. In the future, unmanned air vehicles, such as the "dragon eye," may scout areas or regions for target

species. Such devices could provide photographic recordings of species distribution. Sensors may remotely register contents of specifically desirable natural products and may map compound-rich locations from the air. Hand-released "spider" robots may be deployed to the tops of trees or high ridges to collect and retrieve inflorescences or fruit.

Do's and Don'ts

Do's

- Get acquainted with the International Code of Conduct for Plant Germplasm Collecting and Transfer of FAO (1993).
- Always keep a route map of the target area with list of important places and the distance covered during travel to facilitate report writing.
- Before entering into a forest take the help of forest guards to have forehand knowledge of possible dangers in the target area. If needed, help of a gunman is taken during survey in dense forest.
- Explain the purpose and get consent from the farmers for collecting germplasm**.**
- Keep important telephone numbers of concerned officers including district authorities, hospitals, dispensaries and police station.
- Keep your identity card and a certificate from Head of Organization for proposed mission.
- Honour social customs of local inhabitants of the target area.
- While talking and discussing with ladies, be polite and respectful to them
- After day's collection and before retiring to bed, have a glance at your equipments, passport data and collected material for need-based updating.

Don'ts

- Do not provide lift to strangers in your vehicle under any pretext.
- Do not indulge in unnecessary discussion related to politics, religion and local beliefs with the local people.
- Do not make false promises with donors.
- Do not plan the expedition during important festivals and peak election campaign in the target area.
- Do not enter any house for seed collection in absence of male members of the family.
- Do not eat unknown wild fruits since some of them may be toxic or internally infected.
- Do not collect the seed in large quantities from any household if the farmers wish so.

- Over-collecting of the genetic diversity with similar attributes should be avoided to save time and energy in collection and evaluation and to save space in the genebank.

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Annexure I

NATIONAL BUREAU OF PLANT GENETIC RESOURCES, NEW DELHI -12

PASSPORT DATA SHEET

Date.....Collector's No.....Accession No.....
Botanical Name..... Common Name (English)..... Crop/Vern. Name.....
Cultivar name.....Region Explored..... Village/Block.....
District.....State.....Latitude…..……^{0}N
Longitude…..……^{0}E Altitude…..……..m Temp..... Rainfall .....

COLLECTION SITE	1. Natural wild 2.Disturbed wild 3.Farmer's field 4.Threshing yard 5.Fallow 6.Farm store 7.Market 8.Garden 9.Institute 10.....
OGICAL STATUS	1.Wild 2.Weed 3.Landrace 4.Primitive cultivar 5.Breeder's line
FREQUENCY	1.Abundant 2.Frequent 3.Occassional 4.Rare
MATERIAL	1. Seeds 2.Fruits 3.Inflorescence 4.Roots 5.Tubers 6.Rhizomes 7. Suckers 8.Live plants 9.Herbarium 10……..
BREEDING SYSTEM	1.Self-pollinated 2.Cross-pollinated 3.Vegetatively propagated
SAMPLE TYPE	1.Population 2.Pure line 3.Individual plant
SAMPLE METHOD	1.Bulk 2.Random 3.Selective (non-random)
HABITAT	1. Cultivated 2.Disturbed 3.Partly disturbed 4.Rangeland 5……..
DISEASE SYMPTOMS	1.Susceptible 2.Mildly susceptible 3.Tolerant 4.Resistant 5.Immune
INSECT/ PEST/ NEMATODE INFECTION	1. Mild 2.Moderate 3. High
CULTURAL PRACTICE	1. Irrigated 2.Rainfed 3.Arid 4.Wet 5.....
SEASON	1.Kharif 2.Rabi 3.Spring-summer 4.Perennial type
ASSOCIATED FLORA	1. Sole 2.Mixed with……
SOIL COLOUR	1. Black 2.Yellow 3.Red 4.Brown 5……..
SOIL TEXTURE	1.Sandy 2.Sandy loam 3.Loam 4.Silt loam 5.Clay 6.Silt
TOPOGRAPHY	1.Swamp 2.Flood plain 3.Level 4.Undulating 5.Hilly dissected 6.Steeply dissected 7.Mountainous 8.Valley
AGRONOMIC SCORE	1.V poor 2.Poor 3.Average 4.Good 5.V good
ETHNOBOTANICAL USES	
PART(S)	1.Stem 2. Leaf 3.Root 4. Fruit 5.Flower 6.Whole plant 7.Seed 8.Others
KIND	1.Food 2.Medicine 3.Fibre 4.Timber 5.Fodder 6.Fuel 7.Insecticide/ Pesticide 8.Others
HOW USED	.....
INFORMANT(S)	1.Local Vaidya 2.Housewife 3.Old folk 4.Graziers /Shepherds 5.Others
PHOTOGRAPH	1.Colour/Video
FARMER'S/ DONOR'S NAME.....ETHNIC GROUP.....	
ADDRESS	
PLANT CHARACTERISTICS/ USES ADDL. NOTES	

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

PASSPORT DATA FORM

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 issuance of 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: SPA/KP/231 (expanded form is: collector number assigned in an exploration by SP Ahlawat 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/ 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 (75^0 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.

5

International and National Regulation Governing the Use of Plant Genetic Resources

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Plant genetics resources (PGR) refer to the diversity available in plants that are nurtured by humans and that are nurturing the humans and domesticated animals. PGR are essential for agricultural development and growth. They include all plants species of actual or potential value and contribute to the livelihood of people, provide food, nutrition, medicine, feed for domesticated animals, fiber clothing, shelter, energy and other uses. PGR provide the raw material for crop improvement and help adapt to ever changing climate through development of adoptive verities. For this very purpose, availability of PGR to the community of researchers and breeders is of paramount importance. Their access and utilization is crucial for world's food and nutritional requirements. PGR are acquired by either collecting the crop diversity from the farmers' fields or from the centers of diversity of crop plants distributed throughout the world. No country or organization is self-sufficient in its PGR requirements. Certain principals govern the exchange and continued availability of PGR. Before the plant breeders rights (by Private sector initially) came into being, PGR were freely available and were considered the 'heritage of mankind'. As and when bred verities were restricted for exchange by some by big private companies, and countries where private sector become a major player, the availability of PGR become restricted for further breeding. On the other hand, the developed countries and bigger companies did not share the equitable profits after commercialization of the biological resources, with the provider of these resources, which were farmers or traditional knowledge holders mostly in developing countries. This historical inequity, popularly known as 'bio piracy' led to the negotiations of certain international conventional and agreements, that affect the current scenario of regulations for sharing and exchange of PGR at national and international land. These are described below:

International Regulations

Convention on Biological Diversity (CBD)

CBD is the most important convention to understand by all dealing with biological resources. It is applicable to all of biodiversity and provides general principles for access and benefit-sharing concerning materials collected after the coming of CBD (and not covered by the International Treaty on PGRFA). It came into force in December 1993. It is legally binding for countries that have ratified (196 countries as on 2019). All parties/countries must adopt appropriate legislation/regulations and/or bring existing ones into harmony with the

Convention. The objetives of the CBD the are conservation of all components of biodiversity; sustainable use of the components of biodiversity; and sharing of benefits arising from the utilization of geneticresources in fair and equitable way. The access to the biological resources is to be determined by national governments subject to the following principles that are:

- Prior Informed Consent (PIC)
- Mutually Agreed Terms (MAT).

The genetic resources occuring in their jurisdictions are to be managed by the countries themselves, and CBD threfore declared them as soverign rights of the Nations. The right to provide access and the conditions of access are determined by individual countries, depending on the nature and purpose of Access.

International Treaty on Plant Genetic Resources for Food and Agriculture (ITPGRFA)

The ITPGRFA is a legally binding agreement adopted by FAO Conference in 2001 it came into force after the 40th country ratified in November 2004. It is legally binding for all countries that ratify and not applicable to non-member countries. Countries that ratify are required to bring national laws and regulations into conformity with the Treaty. The objectives of ITPGRFA are same as the CBD accept that it applies to PGRFA only.

It covers all PGRFA and addresses diverse topics, including conservation, use, international cooperation, technical assistance and farmers' rights. The Treaty has established a multilateral system for selected crops (over 35 food crop/29 forages species), and has sets rule for access and benefits- sharing for these materials, both ex situ and in situ, while respecting property rights. The Treaty does not regulate the non-food and non-agricultural uses of the PGFRA as defined in the purpose of access from the multilateral system of the Treaty. Most of international crop research institutions known as the CGIAR Centers have also signed an agreements with the Treaty's Governing Body in order to adhere to the Treaty. These collections, which were available with them prior to the CBD, are now formally into FAO trust. These are freely available to all under the conditions of the Standard Material Transfer Agreement.

International Plant Protection Convention

IPPC is an instrument and forum for harmonizing Phytosanitary measures used in regulating international trade. It provides for international regulatory framework for trade and protection of environment from adverse effects of products of modern biotechnology. It was established in 1952 (last revised in 1997) and adopted in January 2000. Regular updating is done through this forum in case significant development in the Phytosanitary measures of international importance.

CBD/International Protocol on Biosafety (Cartagena Protocol)

This agreement provides for specific the set of guidelines for trans- boundary movement living modified organism (LMOs). While exchanging the LMOs, the member States must comply with minimum standards of protection of IP. The safe and secure use of LMOs is the equal responsibility of the importing countries and they must frame guidelines for handling, storage and utilization of the LMOs.

WTO-TRIPS Agreement 2005

TRIPS (Trade related aspects of intellectual property rights) agreement is part of World Trade Organization, which affects use of biological resources. It mainly concern patenting or other rights such as plant variety protection on Geographical Appellations. It entered into force in January 1995 after the Marrakesh Ministerial Meeting in April 1994. It is legally binding on all WTO Members. It provides for possibility for trade sanctions for those found not in compliance. As per this Agreement, the countries are required to ensure protection of microorganisms, non- biological and microbiological processes and plant varieties that meet protection criteria of protection or registrations.

WTO/Agreement on Application of Sanitary and Phytosanitary Measures

The SPS agreement covers measures adopted by countries to protect human or animal life from foodborne risks: animal or plant-carried diseases; plant pests and diseases to ensure food safety, prevent the spread of pests among animals, and plants. 167 WTO members have adopted it presently. The food and feed exports are to be free from pest and diseases, which affect human an animal health in the importing country.

Union for Protection of Plant Varieties (UPOV)

UPOV aims to maximize plant-breeding efforts by proving a model for securing protection under UPOV for plant breeders' rights for plant varieties. Countries signatory to the UPOV have their national laws as per the guidelines for the plant variety protection. Four versions of UPOV are available until date. India is not a signatory to UPOV.

FAO Global plan of Action

Adopted in 1996 by the 4th Intl Technical Conference on PGRFA, legally non-binding and served as a framework, guide and catalyst for PGRFA. Countries can manage their PGRFA as per the framework of GPA and assign their strength and weaknesses and would their future activities.

It covers all PGRFA and contains specific "activates" on *in situ* conservation and development, *ex situ* conservation, utilization and institutions and capacity building. Although it is a non-legal framework, it is a supporting component of the ITPGRA, which is legally binding and thus has bearing a relation to the Treaty.

FAO Code of Conduct for Plant Germplasm Collecting and Transfer

This is a voluntary code which deals with the etiquettes of collecting and transfer of PGRFA and it has as a provision on collectors, sponsors, curators and users, as well as on reporting, monitoring and evaluating observance of the Code. This was adopted by FAO Conference in 1993 not legally binding.

Nagoya Protocol on Access and Benefit Sharing Entered into Force: 12 October 2014

The International Regime on Access and Benefit sharing popularly known as the Nagoya Protocol, 2012. It was negotiated under the CBD, in order to provide greater legal certainty and transparency for both providers and users of genetic resources and associated traditional knowledge by: establishing more predictable conditions for access to those resources; and Helping to ensure benefit-sharing when genetic resources leave the contracting Party providing the genetic resources. The Access and Benefit-sharing Clearing-House (ABS Clearing-House, ABSCH), a platform for exchanging information on access and benefit-sharing established by Article 14 of the Nagoya Protocol, as part of clearing-house mechanism under Article 18, paragraph 3 of CBD.

The ABS Clearing-House is a key tool for facilitating the implementation of the Nagoya Protocol by enhancing legal certainty, clarity and transparency on procedures for access and for monitoring the utilization of genetic resources along the value chain. By making relevant information regarding ABS available, the ABS Clearing-House helps users access genetic resources and associated traditional knowledge, and providers fairly and equitably share in the benefits arising from their utilization.

National legislations with implication on access to plant genetic resources

Acts/Guidelines

The Biological Diversity Act, Effective from: April 2004 (CBD)

Conservation and sustainable use of biological diversity; regulation of access to biological resources; secure sharing of benefits out of use if Indian biological resources/ use of traditional knowledge. The access to Indian biological resources by non-Indians shall be subject to the approval of National Biodiversity Authority. These conditions do not apply to transfer of genetic resources under any project approved by the Government of India, and transfer of results under approved projects.

Protection of Plant Varieties and Farmer Rights (PPVFR Act, 2001) Effective from: January 2005. (WTO- TRIPS)

It is to provide for an effective system of protection of plant varieties: and right of farmers and plant breeding; ensure availability of high quality seeds improved varieties to farmers.

New varieties to be protected (registered) in the Act, should be novel, distinct, and stable and uniform (NDUS), and shall be subjected to tests by the PPVFR Authority. Application for new varieties should be accomplished with complete passport data of all the genetic resources used in the development of new material. Proof of lawful acquisition of all materials used in development of new varieties to be furnished.

Patent (Amendments) Act 2005, Effective from: January 2005 (WTO- TRIPS Agreement)

New amendment include patenting on newly developed (GM) micro-organisms and micro biological process that conform to the criteria of novelty, inventive step and utility. Sources of biological resources used in the development of patents is to be acknowledged. All biological resources used in development of patents to be deposited in the designated repositories before sealing of patents.

Geographical Indications of Goods (Registration and Protection) Act 1999 (WTO- TRIPS Agreement)

Applicable to goods such as agricultural goods, natural goods or manufactured goods as originating and manufactured in a territory of a country Where the given quality/ reputation is essentially attributable its geographic region (Land races of crop plants specific to certain agro-climatic regions of the country).

Plant Quarantine (Regulation of Import into India) Order 2003 under the DIP Act 1914, Effective from: April, 2004 (IPPC, SPS-WTO)

Rules for Regulating the Import of Plants etc. into India, including import of germplasm/genetically modified organisms (GMOs/transgenic planting material; live insects/ fungi including bio-control agents, soil, peat, and sphagnum moss, timber and wooden logs. The Order includes prohibition on import of commodities with weed/alien species contamination and restriction on import of packaging material of plant origin unless treated. Additional declarations to be given on Phytosanitary certificates have been specified and notified points of entry have been increased.

Guidelines for Safety of Biotechnology by DBT under the EPA 1986, Effective from: 1990 (last revised in 1998) (Cartagena Protocol- CBD)

It is a National framework for development of genetically modified plants and their commercial exploitation. It gives guidelines for import and shipment of genetically modified plants only for toxicity and allergen city evaluation of transgenic seeds, plants and plant parts.

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http://www.plantauthority.gov.in/ (PPVFRA)

http://www.ipindia.nic.in/acts-patents.htm (Patent Act)

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6

Introduction and Exchange of Plant Genetic Resources

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Diversity in plant genetic resources (PGR) is the key to sustainable agriculture. There are various sources of diversity including natural variation, created variation through hybridization, mutation etc. or through introduction. Plant introduction gives new opportunities to enrich the plant wealth of the country which may be tapped for utilization in breeding programme. Introduced crop may be an entirely new crop plant, a new variety, or a germplasm for scientific/research studies. Since times immemorial, crop plants have traveled into new areas from their centers of origin through travelers, settlers, traders, invaders, explorers, pilgrimists and naturalists, knowingly or unknowingly. Some of the notable examples are introduction of cherries and grapes from Afghanistan by *Muslim invaders*(1300 A.D.); maize, groundnut, chillies, potato, sweet potato, guava, pineapples, papaya, cashew nut and tobacco by *Portugese* (17^{th} Century); tea, litchi, loquat from China by *East India Company*; annato, mahogany from W. Indies in last quarter of 18^{th} Century. Crops like mustard, pear, pomegranate, apple and walnut have been introduced from the Central Asia during the last five centuries. These introductions had tremendous impact on Indian agriculture. In the 19^{th} Century, botanical gardens played important role in introduction of quinine and rubber trees. Later agricultural and horticultural research stations were established in the country for introduction of horticultural and agricultural crops. Germplasm Exchange is the mutual give and take of germplasm or PGR from all available sources. It includes import, export and national or domestic supply of PGR. ICAR-NBPGR is the nodal agency to look after these activities.

Introduction/Import of PGR

Plant Introduction is defined as taking a genotype or a group of genotypes of plants into new environments where they were not being grown before. Often the term is used for introducing the material from other countries, but movement of crop varieties from one environment into another within the country is also termed as introduction. Important crops like maize, potato, tomato, tobacco, soybean, sunflower, kiwi fruit, tree tomato, hazel nut, guayle, jojoba, geranium are examples of new crop introductions. When the introduced variety is well suited to the new environment and is released for the commercial cultivation without any alteration in the original genotype it is termed as primary introduction, while when the introduced variety is subjected to selection to isolate a superior variety or is hybridized with local varieties to transfer one or few characters from this variety to the local ones, this is known as

secondary introduction. Introduction of semi-dwarf wheat varieties Sonora 64, Lerma Rojo from CIMMYT, Mexico; and rice varieties Taichung Native 1, IR 8, 1R 28, 1R 36 from IRRI, Philippines are some examples of primary introduction and wheat varieties Kalyan Sona, Sonalika, selected from material introduced from CIMMYT, Mexico are examples of secondary introduction. Though introduction is very important we need to have a proper pest risk analysis system to obviate the risk of introducing associated pests and diseases along with the plants. Several pests and diseases including Late blight of potato, Flag smut of wheat, Bunchy top of banana, Wooly aphid and fluted scale of citrus has been reported to be introduced with the imported material.

Plant introduction services have been established in many countries for systematic introduction. These services have some features in common which facilitate their efficient operation such as: they usually form part of the department of agriculture or a major research organization to draw upon extensive resources; has direct responsibility for plant quarantine and work in close collaboration with the quarantine authority; have good testing facilities in all the major climatic zones; maintain records of introduced plants and provides a contact point for the international collaboration through FAO and other agencies.

How the journey of Introduction started in India

Centralized Plant Introduction agency was initiated in 1946 at Indian Agricultural Research Institute (IARI) in the Division of Botany, New Delhi which was expanded as Plant Introduction and Exploration organization in 1956 under second five year plan and further upgraded into an independent Division of Plant Introduction, of IARI, New Delhi in 1961. In 1976, it was upgraded to an independent institute as National Bureau of Plant Introduction (NBPI) and in 1977 renamed as National Bureau of Plant Genetic Resources (NBPGR). The ICAR-NBPGR, as a single window system, is responsible for introduction/ import and maintenance of germplasm of agri-horticultural crops. Central Research Institute for various crops *e.g.* tea, coffee, sugarcane, potato, tobacco, cassava, coconut, rubber *etc.* introduce, test and maintain plant material of interest, but their activities are coordinated by ICAR- NBPGR which has the ultimate responsibility for introduction activities. PGR may also be introduced by individual Scientist, Universities and other research organization, but all the introductions in India must be routed through the NBPGR, New Delhi.

Importing PGR for research use

In India, import of any seed/planting material for research purposes in small quantities is governed by Plant Quarantine Order, 2003 or PQ Order, 2003 (www.plantquarantineindia .org). As per this order, we need to fulfill two mandatory requirements before importing any seed/planting material from other countries. These are 1) Import permit issued by ICAR-NBPGR before import of any material (IP) and 2) Phytosanitary certificate from the country of origin (PC). These two documents must accompany every seed/planting material consignment imported from abroad.

Issuance of import permit

As per clause 6 (2) of Plant Quarantine Order 2003, Director, ICAR-NBPGR has been authorized to issue import permit for import of germplasm, transgenic or genetically modified organisms for research purposes and receive imported materials from custom authorities for its quarantine inspection and clearance and further distribution to the researchers in the country. Along with the application form PQ08, processing fee for the issuance of IP should be sent (Annexure 1). The fee is nonrefundable. It should be ensured that the consignment be always be addressed to the Director, NBPGR, New Delhi. Quarantine examination charges and handling fee per consignment may be accessed from ICAR-NBPGR website www.nbpgr.ernet.in. The IP is issued in form PQ 09 in triplicate and is valid for six months from the date of issue and valid for successive shipment provided the exporter and importer, bill of entry, country of origin and phytosanitary certificate are the same for the entire consignment. Validity may be extended up to one year on request, if adequate reasons in writing are justified. Import permit is non-transferable. After obtaining import permit the applicant should send it to the concerned source that has agreed to supply the required germplasm with the request that the import permit in duplicate must be enclosed along with the seed/planting material. Private Seed Companies are required to submit a certificate ensuring their research and development activity is recognized by Department of Scientific and Industrial Research (DSIR). However, under the provisions of Clause 3(3) (4), commercial import of consignments of seeds of coarse cereals, pulses, oil seeds and fodder seeds and seeds/stock material of fruit plant species for propagation shall only be permitted based on the recommendations of EXIM Committee of Department of Agriculture, Cooperation and Farmers Welfare, except the trial material of the same as specified in Schedule-XII of Plant Quarantine Order. Every application for permit shall be made to Plant Protection Advisor or to issuing Authority as listed in schedule X of PQ Order, 2003 in from PQ 01 for the import of plants and plant products for consumption and processing and in from PQ 02 for imports of seeds and plants for propagation covered under schedule V and VI.

Phytosanitary Certificate

The second mandatory requirement is that of Phytosanitary certificate (PC). PC is a document regarding the health status of consignment and issued by Government Official from country of origin in the prescribed format of Food and Agriculture Organization (FAO). Every consignment should be accompanied by PC (original copy) issued by authorized officer at country of origin/ supplier country with additional declarations for freedom from specific pests and diseases as specified or that the pests specified do not occur in the country or state of origin as supported by documentary evidence thereof. PC is also issued by the ICAR-NBPGR for all the germplasm material meant for export to foreign countries.

Import of Transgenics

Gazette of India extra ordinary Part II Section -3 sub section (1) published by authority No. 621, New Delhi has defined the Rules for the manufacture, use, import, export and storage of hazardous microorganism/ genetically engineered organisms or cells made under sections 6,8 and 25 of the Environment (Protection) Act, EPA 1986 (29 of 1986). These rules are applicable for importing any transgenic seed/planting material into the country. EPA plays a major role in minimizing the risk from pollutants and contaminants affecting flora and fauna, human and animal health and preserving the environment. In accordance with this Act, all transgenic plants are regulated items. The provisions of PQ Order, 2003 are applicable to import of transgenic seeds as well. Department of Biotechnology (DBT), under Ministry of Science and Technology and the Ministry of Environment, Forests and Climate Change (MoEFCC) has a set of prescribed procedures for providing permission for import of transgenics. Proposals for import of transgenics are submitted to Review Committee on Genetic Manipulation (RCGM) through the Institutional Biosafety Committee (IBSC). RCGM is an authorized agency of the Government of India, functioning under DBT, which assesses the applications submitted for importing transgenic material for research purposes and issues Seed Transfer Clearance Letter. RCGM examines the desirability of import of transgenic line, from the biosafety point of view. It includes representatives from DBT, Indian Council of Medical Sciences (ICMR), Indian Council of Agricultural Research (ICAR), Council of Scientific and Industrial Research (CSIR) and other experts in their individual capacity. After getting the technical clearance for Seed Transfer Clearance Letter issued by DBT, application for issuance of IP is submitted to the Director, ICAR-NBPGR in the prescribed PQ08 form. Complete Procedure for import of PGR is depicted in Annexure 3.

Quantity of seed permitted for import

Small quantity of seed/planting material can be imported for research purposes. The quantity of seed or planting material just sufficient for the establishment of plant is considered the optimum and safest (Annexure 2). For transgenic the seed quantity is specified in DBT clearance letter. Five to ten percent of the seed/ planting material is retained in the National Genebank as voucher specimen for reference purposes. Seed quantity permitted for import and export is presented as Annexure 2.

Export of PGR

Under the provisions of the Convention on Biological Diversity (CBD), Government of India enacted legislation called Biological Diversity Act (BDA), 2002, and also notified the Biological Diversity Rules, 2004 which suggests that no person from outside India or a body corporate, association, organization incorporated or registered in India having non-Indian participation in its share capital or management, can access any biological resources or knowledge associated, for research, commercial utilization, bio-prospecting or bio-utilization, without prior approval of National Biodiversity Authority (NBA). However, as per Section 5

of BDA, 2002, exchange of germplasm for collaborative research projects which confirm to the policy guidelines issued by the Central Government or approved by the Central Government are exempted from Section 3 and 4 of BDA, 2002.The request for access to germplasm occurring in India, only for research purposes is categorized under the four categories:

Export of germplasm under collaborative research projects, under Section 5 of the BDA, 2002

1) Export of Annex 1 crops under International Treaty on Plant Genetic Resources (ITPGRFA) and FAO designated accessions of CG Centres located in India.
2) Indian researcher/ Government institution to carry/send germplasm for non-commercial research/ or research for emergency purposes other than collaborative research.
3) Export of germplasm not covered in any of the above category that is access by non-Indian entity for any biological resource occurring in India which is not Annex I crop under ITPGRFA neither covered under any collaborative research project.

For export of PGR under collaborative research projects which are compliant to MOEFCC Guidelines as per provisions of Section 5 of the BDA, 2002, ICAR-NBPGR facilitates the procedure for approval of export from the Competent Authority. For requests of Annex 1 crops under ITPGRFA and FAO designated accessions of CG Centres located in India (ICRISAT), ICAR-NBPGR facilitates the process for approval of the Competent Authority *i.e.* Department of Agricultural Research and Education (DARE) export of germplasm under Collaborative Research Project and Department of Agricultural Cooperation and Farmers Welfare (DAC&FW) for export under ITPGRFA. For all other cases, the applicant is required to contact NBA for seeking approval (Category 3 or 4). Once NBA grants approval for export the PC shall be issued by Division of Plant Quarantine after quarantine inspection. Procedure for export of PGR is depicted in the flow chart (Annexure 4).

Domestic supply of PGR

The researchers /users desirous of obtaining any seed/ planting material for research/ experimental purpose from ICAR-NBPGR may submit the request in the prescribed requisition proforma (GEX 01), along with duly filled and signed Material Transfer Agreement (MTA). Both the forms (GEX 01 and MTA) are available on NBPGR website (www.nbpgr.ernet.in). The private seed companies are required to submit certificate of R & D from DSIR.

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http://www.nbpgr.ernet.in (NBPGR)

www.plantquarantine.india.org (PQ Order)

http://dbtindia.nic.in/uniquepage.asp (DBT, India)

http://www.nbaindia.org/contact.htm (NBA, Chennai)

http://www.planttreaty.org/ (ITPGRFA, FAO)

http://www.biodiv.org/doc/legal/cbd-en.pdf full text of the Convention

Annexure 1

Processing fee applicable for issuance of Import permit *w.e.f.* July 2017

S.No.	Organization/ Institution*	Import Permit	Fee amount in Rs.	GST @18%	Total Fee per permit in Rs.
1.	Public	Non transgenic	150.00	27.00	177.00
2.	Public	Transgenic	250.00	45.00	295.00
3.	Private	Non transgenic	300.00	54.00	354.00
4.	Private	Transgenic	550.00	99.00	649.00

Fee to be paid as Demand Draft in favour of Director, ICAR-NBPGR payable at Delhi

Revalidation fee for Extension of validity or revision of Import Permit

S. No.	Category	Public Sector	Private Sector
1.	Revalidation of existing Import Permit for a further period of six months for import of non-transgenic germplasm	Rs. 25/-	Rs. 50/-
2.	Revalidation of existing Import Permit for a further period of six months for import of transgenic germplasm	Rs. 50/-	Rs. 100/-

***Organizations/ Institution qualifying for import of germplasm/ research material**

The government notifications recognizing the R&D status of an organization as issued by the Department of Scientific and Industrial Research/ Department of Science and Technology/ State Government Department (s), will be considered as the criterion for issuing the import permit to a given organization.

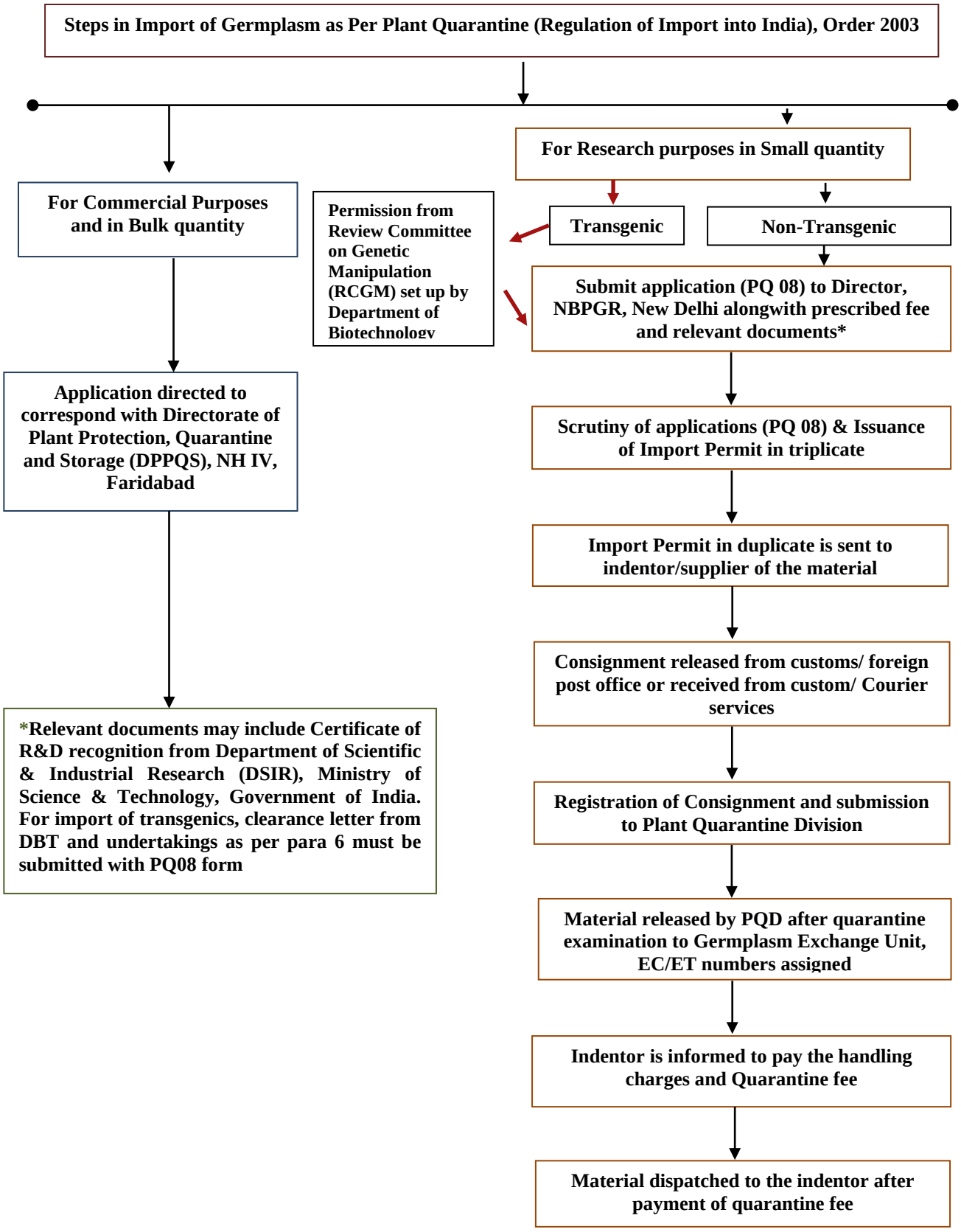
**** GST @ 18% applicable (subject to revision)***

Annexure 2

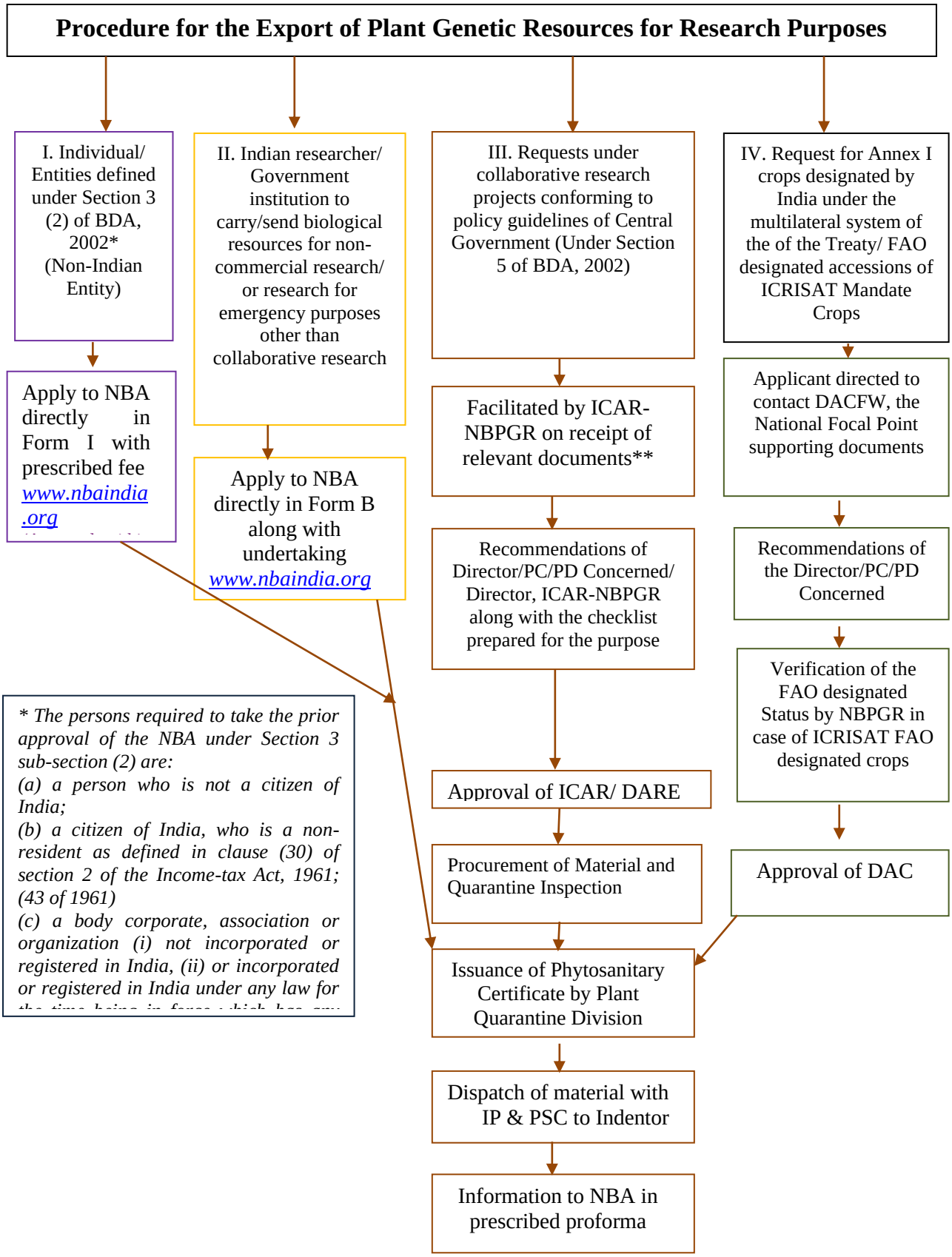
Maximum quantity permitted for Import and Export, per sample/accession/variety for research purposes

S.No.	Plants parts	Quantity Permitted per sample/ accession/ variety
1.	**Seed**	
	Large seeded crop species *viz; Zea mays*, *Helianthus* spp, *Carthamus* spp. *Phaseolus* spp. *Arachis* spp., *Dolichos* spp., *Mucuna* spp., *Pisum sativum*, *Cicer* sp., *Vicia* spp., *Cajanus* spp., *Canavalia* spp., *Cyamopsis* spp., Palms and others	**Up to 1kg**
	Small seeded crop species *viz; Allium* spp; *Brassica* spp; *Capsicum* spp; *Solanum melongena*, *Carica papaya* and others	**Up to 100g**
	Very small/ light weight seeded crop species *viz;* Tobacco, Tomato, Grasses, Eucalyptus etc.	**Up to 25g**
	All other species of plants viz; Rice, Wheat, Barley *etc*	**Up to 200g**
2.	**Vegetative Propagules**	
	Number of rooted cuttings/ plants	**Up to 25 in numbers**
	Number of other vegetative propagules	**Up to 50 in numbers**

Annexure 3


Steps in Import of Germplasm as Per Plant Quarantine (Regulation of Import into India), Order 2003
For Commercial Purposes and in Bulk quantity
For Research purposes in Small quantity
Permission from Review Committee on Genetic Manipulation (RCGM) set up by Department of Biotechnology
Transgenic
Non-Transgenic
Submit application (PQ 08) to Director, NBPGR, New Delhi alongwith prescribed fee and relevant documents*
Application directed to correspond with Directorate of Plant Protection, Quarantine and Storage (DPPQS), NH IV, Faridabad
Scrutiny of applications (PQ 08) & Issuance of Import Permit in triplicate
Import Permit in duplicate is sent to indentor/supplier of the material
Consignment released from customs/ foreign post office or received from custom/ Courier services
Registration of Consignment and submission to Plant Quarantine Division
Material released by PQD after quarantine examination to Germplasm Exchange Unit, EC/ET numbers assigned
Indentor is informed to pay the handling charges and Quarantine fee
Material dispatched to the indentor after payment of quarantine fee
*Relevant documents may include Certificate of R&D recognition from Department of Scientific & Industrial Research (DSIR), Ministry of Science & Technology, Government of India. For import of transgenics, clearance letter from DBT and undertakings as per para 6 must be submitted with PQ08 form

Annexure 4


Procedure for the Export of Plant Genetic Resources for Research Purposes
I. Individual/ Entities defined under Section 3 (2) of BDA, 2002* (Non-Indian Entity)
II. Indian researcher/ Government institution to carry/send biological resources for non-commercial research/ or research for emergency purposes other than collaborative research
III. Requests under collaborative research projects conforming to policy guidelines of Central Government (Under Section 5 of BDA, 2002)
IV. Request for Annex I crops designated by India under the multilateral system of the of the Treaty/ FAO designated accessions of ICRISAT Mandate Crops
Apply to NBA directly in Form I with prescribed fee www.nbaindia.org
Apply to NBA directly in Form B along with undertaking www.nbaindia.org
Facilitated by ICAR-NBPGR on receipt of relevant documents**
Applicant directed to contact DACFW, the National Focal Point supporting documents
Recommendations of Director/PC/PD Concerned/ Director, ICAR-NBPGR along with the checklist prepared for the purpose
Recommendations of the Director/PC/PD Concerned
Verification of the FAO designated Status by NBPGR in case of ICRISAT FAO designated crops
Approval of ICAR/ DARE
Approval of DAC
Procurement of Material and Quarantine Inspection
Issuance of Phytosanitary Certificate by Plant Quarantine Division
Dispatch of material with IP & PSC to Indentor
Information to NBA in prescribed proforma
* The persons required to take the prior approval of the NBA under Section 3 sub-section (2) are:
(a) a person who is not a citizen of India;
(b) a citizen of India, who is a non-resident as defined in clause (30) of section 2 of the Income-tax Act, 1961; (43 of 1961)
(c) a body corporate, association or organization (i) not incorporated or registered in India, (ii) or incorporated or registered in India under any law for

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Principles of Plant Quarantine and National Quarantine Set-up in India

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Plant quarantine is a government endeavour enforced through legislative measures to regulate the introduction of planting materials, plant products, soil, living organisms, etc. in order to prevent inadvertent introduction of pests (including fungi, bacteria, viruses, nematodes, insects and weeds) harmful to the agriculture of a country/ state/ region, and if introduced, prevent their establishment and further spread. The plant quarantine measures act as filters against the entry of exotic species and check or delay the introduction of unwanted organisms. Therefore, practicing plant quarantine i.e. protection of plants by observing quarantine measures will go a long way in protecting our agriculture from the ravages of exotic pests and diseases and also from further spread of those present in a particular region.

Plant quarantine measures are implemented at different levels of authority:

1. Legislation enacted by the national authority and in some cases by the state authority under which rules and regulations are promulgated and which are amended keeping in view the needs as per the time and international developments.
2. To enable the legislation the government authority is empowered which is usually the Ministry of Agriculture to issue the notifications or directives which are themselves rules for implementation.
3. The network of quarantine implementation authorities which ensure that the rules are followed.

In case the country is a member of a larger inter-governmental organization, rules are there that are binding to all member countries. For instance, the Asia Pacific Plant Protection Commission (APPPC) has >25 member countries and the European and Mediterranean Plant Protection Organization has >35 members.

The word quarantine has its roots in the Latin word for forty. It originally referred to the period of detention which was imposed on ships' passengers to allow latent cases of disease to develop before passengers were permitted to land. The earliest record of such restrictions for human disease goes back to the latter half of the 14th century. Subsequently, as governments became more concerned with the spread of pests destructive to agricultural and forest crops, new

controls were gradually introduced under the name Plant Quarantine (Khetarpal and Gupta, 2008).

The first plant quarantine law was promulgated in Rouen, France in 1860 to suppress and prevent the spread of common barberry, which is the alternate host for wheat rust. Other countries to establish the quarantine laws after France were Germany, USA, UK and Australia. In 1881, a California Pest Act was passed to prevent the spread of grape Phylloxera. However, subsequently, a number of countries became aware of the necessity and importance of plant quarantine for the safety and health of their agriculture. The first effort towards an International Agreement on Plant Protection was made in 1914 under the auspices of the International Institute of Agriculture, Rome. This was followed by an International Convention of Plant Protection with over 50 member countries of the Institute in 1919 under which Agreements regarding the issue and acceptance of phytosanitary certificates were finalized. The project got a set back due to Second World War and was later appreciated by Food and Agriculture Organization (FAO) and in 1951, an International Plant Protection Convention (IPPC) was convened by the FAO (https://www.ippc.int/IPP/En/default.jsp). India became a party to this agreement in the year 1956 along with Australia, Sri Lanka, UK, Netherlands, Indonesia, Portugal and Vietnam. At present there are 181 signatory members of the IPPC.

The International Plant Protection Convention (1952) was initiated with the aim of developing international cooperation among various countries in preventing the introduction and spread of pests across national boundaries to secure common and effective measures to this end. This convention is applicable mainly to the regulated pests involved with international movement of plants and planting material.

National Plant Protection Organization

A National Plant Protection Organization (NPPO) is an official service established by a government to fulfil the functions specified in the IPPC. NPPOs implement the phytosanitary laws and/or regulations issued by their governments. Responsibilities of NPPOs under the IPPC (Article IV) include:

- issuance of phytosanitary certificates,
- surveillance and inspection,
- controlling pests (for example, administering treatments, preventing spread, disinfection or disinfestation),
- protecting endangered areas,
- conducting pest risk analyses,
- ensuring phytosanitary security of consignments from certification until export, and
- designation, maintenance and surveillance of pest free areas and areas of low pest prevalence.

To facilitate information exchange between the IPPC and contracting parties, each country has a designated official contact point, which is most often the NPPO. These contact points can be found on the IPPC web site: https://www.ippc.int/IPP/En/nppo.jsp. In India, the Directorate of Plant Protection Quarantine and Storage, Faridabad is the NPPO.

Principles of Plant Quarantine as Related to International Trade

The IPPC through its International Standard on Phytosanitary Measures 1 on the above aspect was formulated with an aim to facilitate the process of developing international standards for plant quarantine the implementation of which would result in elimination of the use of unjustifiable phytosanitary measures as barriers to trade. The general principles indicate the process of development of phytosanitary measures as applicable to international commerce, while the specific principles either directly support the IPPC or are related to procedures in plant quarantine (https://www.ippc.int/servlet/CDSServlet?status=ND0xMzI5MiY2PWVuJjMzPSomMzc9a29z).

General Principles

1. Sovereignty: With the aim of preventing the introduction of quarantine pests into their territories, it is recognized that countries may exercise the sovereign right to utilize phytosanitary measures to regulate the entry of plants and plant products and other materials capable of harbouring plant pests.

2. Necessity: Countries shall institute restrictive measures only where such measures are made necessary by phytosanitary considerations, to prevent the introduction of quarantine pests.

3. Minimal impact: Phytosanitary measures shall be consistent with the pest risk involved, and shall represent the least restrictive measures available which result in the minimum impediment to the international movement of people, commodities and conveyances.

4. Modification: As conditions change, and as new facts become available, phytosanitary measures shall be modified promptly, either by inclusion of prohibitions, restrictions or requirements necessary for their success, or by removal of those found to be unnecessary.

5. Transparency: Countries shall publish and disseminate phytosanitary prohibitions, restrictions and requirements and, on request, make available the rationale for such measures.

6. Harmonization: Phytosanitary measures shall be based, whenever possible, on international standards, guidelines and recommendations, developed within the framework of the IPPC.

7. Equivalence: Countries shall recognize as being equivalent those phytosanitary measures that are not identical but which have the same effect.

8. Dispute settlement: It is preferable that any dispute between two countries regarding phytosanitary measures be resolved at a technical bilateral level. If such a solution cannot be achieved within a reasonable period of time, further action may be undertaken by means of a multilateral settlement system.

Specific Principles

9. Cooperation: Countries shall cooperate to prevent the spread and introduction of quarantine pests, and to promote measures for their official control.

10. Technical authority: Countries shall provide an official Plant Protection Organization.

11. Risk analysis: To determine which pests are quarantine pests and the strength of the measures to be taken against them, countries shall use pest risk analysis methods based on biological and economic evidence and, wherever possible, follow procedures developed within the framework of the IPPC.

12. Managed risk: Because some risk of the introduction of a quarantine pest always exists, countries shall agree to a policy of risk management when formulating phytosanitary measures.

13. Pest free areas: Countries shall recognize the status of areas in which a specific pest does not occur. Upon request, the countries in whose territories the pest free areas lie shall demonstrate this status based, where available, on procedures developed within the framework of the IPPC.

14. Emergency action: Countries may, in the face of a new and/or unexpected phytosanitary situation, take immediate emergency measures on the basis of a preliminary pest risk analysis. Such emergency measures shall be temporary in their application, and their validity will be subjected to a detailed pest risk analysis as soon as possible.

15. Notification of non-compliance: Importing countries shall promptly inform exporting countries of any non-compliance with phytosanitary prohibitions, restrictions or requirements.

16. Non-discrimination: Phytosanitary measures shall be applied without discrimination between countries of the same phytosanitary status, if such countries can demonstrate that they apply identical or equivalent phytosanitary measures in pest management. In the case of a quarantine pest within a country, measures shall be applied without discrimination between domestic and imported consignments.

The IPPC requires that each country establishes a national plant protection organization to discharge the functions specified by it. In India, the Directorate of Plant Protection, Quarantine and Storage (DPPQS) under the Ministry of Agriculture is the body for implementation of plant quarantine regulations as per the IPPC. Over the years, it has worked

under the legislation- Destructive Insects and Pests Act of 1914 which has been amended from time to time as per the requirements.

National Regulatory Mechanism

The awareness to quarantine measures in India started in early 20th century when the Indian Government in 1906 ordered compulsory fumigation of imported cotton bales to prevent introduction of Mexican cotton boll weevil *(Anthonomus grandis*). With a view to restrict the entry of exotic pests, pathogens and weeds through regulation of imports, the Government of India legislated the Destructive Insects and Pests (DIP) Act in 1914 (http://plantquarantineindia.nic.in/pqispub/docfiles/dip_act.htm). This Act has been amended through various notifications issued from time to time also restricted the movement of certain planting material from one state to another state within the country through domestic quarantine. In 1984, a notification was issued under this Act namely Plants, Fruits and Seeds (Regulation of Import into India) Order popularly known as the PFS Order which was revised in 1989 after the announcement of the New Policy on Seed Development by the Government of India in 1988, proposing major modifications for smooth quarantine functioning. This Order has now been superseded by the Plant Quarantine (Regulation for Import into India) Order 2003 which came into force from April 1, 2004 as there was an urgent need to fill-up the gaps in existing PFS order regarding import of germplasm/ GMO's/ transgenic plant material/ bio-control agents etc., to fulfill India's legal obligations under the international Agreements, to protect the interest of the farmers of the country by preventing the entry, establishment and spread of destructive pests, and to safeguard the national bio-diversity from threats of invasions by alien species. Under this Order, the need for incorporation of Additional/ Special declarations for freedom of import commodities from quarantine and invasive alien species (IAS), on the basis of standardized pest risk analysis (PRA), particularly for seed/ planting materials is also dealt with. Further, the scope of plant quarantine activities has been widened with incorporation of additional definitions. The other salient features of the Order are:

- Prohibition on import of commodities with weed/ alien species contamination as per Schedule VIII; & restriction on import of packaging material of plant origin unless treated.
- Provisions included for regulating the import of soil, peat & sphagnum moss; germplasm/ GMOs/ transgenic material for research; live insects/ microbial cultures & biocontrol agents and import of timber & wooden logs.
- Agricultural imports have been classified as (a) prohibited plant species (Schedule IV); (b) restricted species where import permitted only by authorized institutions (Schedule V); (c) restricted species permitted only with additional declarations of freedoms from quarantine/ regulated pests and subject to specified treatment certifications (Schedule VI) and; (d) plant material imported for consumption/ industrial processing permitted with normal Phytosanitary Certificate (Schedule VII).

- Additional declarations being specified in the Order for import of 820 agricultural commodities with specific lists of more than > 1000 quarantine pests and 31 weed species.
- Notified points of entry increased to 130 from the existing 59.
- Certification fee and inspection charges have been rationalized.

Till June 2019, seventy two amendments of the Plant Quarantine (PQ) Order 2003 have been notified to the WTO revising definitions, clarifications regarding specific queries raised by quarantine authorities of various countries, with revised lists of crops under the Schedules IV, V VI and VII. The revised list under Schedule VI and VII now include 698 and 298 crops/ commodities, respectively.

Quarantine Set-up in India

The Directorate of Plant Protection, Quarantine and Storage (DPPQS) of Ministry of Agriculture and Farmers Welfare is the apex body for implementation of plant quarantine regulations and the Plant Quarantine (Regulation of import into India) Order 2003 forms the basis of the functioning of the Directorate. It has a national network of 57 plant quarantine stations at different airports (13), seaports (34) and land frontiers (12). In all, three categories of materials are being imported: (a) bulk consignments of grains/ pulses for consumption, (b) bulk consignments of seeds/ planting materials for sowing/ planting, and (c) samples of germplasm in small quantities for research purposes. The PQ Stations under the DPPQS undertake quarantine processing and clearance of consignments of the first two categories. While ICAR-National Bureau of Plant Genetic Resources (ICAR-NBPGR) undertakes the quarantine processing of all plant germplasm and transgenic planting material under exchange. It has developed well-equipped laboratories and green house complex. A containment facility of CL-4 level has also been established for processing transgenics.

India is unique in its quarantine system, where the National Genebank houses the Quarantine Division for safe exchange of germplasm and a single-window system has been developed for all material being exchanged for research purposes, be it germplasm or trial. This Division works under the delegated powers of the Plant Quarantine Order 2003 of the DPPQS (the National Plant Protection Organization for India).

In India the export/ import of seeds and planting material is governed by the Export-Import Policy 2017-22 which imposes restrictions on export of all cultivated varieties of seeds - breeder/ foundation/ wild varieties; seeds of jute, onion, berseem, cashew, *Nux vomica*, rubber, pepper cuttings, sandalwood, saffron, neem, forestry species and wild ornamental plants with few exceptions. For all other types of seeds/ planting material export restrictions has been removed w.e.f. April 1, 2002.

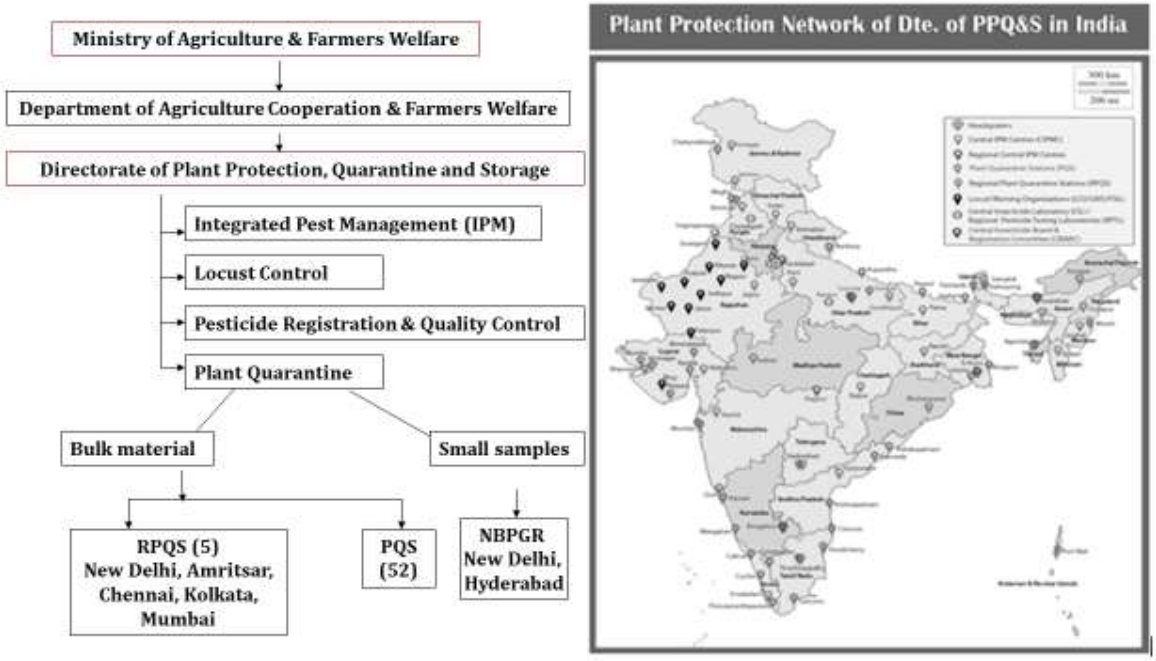

Figure 1: Quarantine Set-up in India

The provisions regarding import of seeds and planting material are:

- Import of seeds/ tubers/ bulbs/ cuttings/ saplings of vegetables, flowers and fruits is allowed without a license in accordance with import permit granted.
- Import of seeds, planting materials and living plants by Indian Council for Agricultural Research (ICAR), etc. is allowed without a license in accordance with conditions specified by the Ministry of Agriculture.
- Import of seeds/ tubers of potato, garlic, fennel, coriander, cumin, etc. is allowed in accordance with import permit granted.
- Import of seeds of wheat, rye, barley, maize, soybean, groundnut, safflower etc. allowed with license subject to New Policy for Seed Development (NPSD) and in accordance with the import permit granted.
- A small quantity of seeds, sought to be imported would be given to ICAR, or farms accredited by ICAR, for trial and evaluation for one crop season.

While importing seeds it would be ensured that there is absolutely no compromise on plant quarantine procedures.

Conclusion

Under the present liberalized trade regime of WTO, the NPPOs and the quarantine personnel have a big responsibility for properly achieving their objectives of excluding the exotic pests or to carry out eradicative measures. The risk of pest introduction and means to stop the

establishment of these pests into new areas continues to be their major concern. Unless proper phytosanitary measures are taken, pests could get transported all over the globe, become established in new areas and devastate agricultural production. On the other hand, there are tremendous opportunities for countries to export of agricultural commodities to boost their national economy if they meet the international quality standards and overcome phytosanitary constraints.

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8

Quarantine Procedures for Exchange of Plant Genetic Resources

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The word quarantine comes from the Italian *quarantigiorni*, meaning 'forty days'. When bubonic plague swept through Europe in the 14th century, the government of Venice required ships to anchor away from the city for 40 days before they could unload passengers or cargo. The authorities thought 40 days would be enough time for any disease to be identified and either treated or pass through its normal course. All ships under quarantine had to fly a yellow flag. Quarantine is designed to prevent the introduction, establishment, or spread of animal, plant or human pests and diseases. All plants or parts of plants such as fruits, seeds, cuttings, bulbs and corms, as well as things made from wood, must be examined and if necessary, treated. Plants/ planting material are tested at special plant quarantine stations when they arrive in Indiato make sure they are not carrying pests or diseases.It is the legislative measure which regulates the introduction of plants and planting material (including the packaging material) into the country to prevent the inadvertent entry/ establishment of the exotic pests (insects, mites, nematodes, fungi, bacteria, viruses and weeds) and also the spread of pests, already established, to other parts of the country.

A quarantine pest is a pest of potential economic importance to the area endangered thereby and not yet present there, or if present, not widely distributed and being officially controlled by appropriate legislation.It plays an important role in preventing introduction of pests along with the exchange of plant/ planting material in a new geographical area. Almost all countries regulate the importation of plants/ planting material because of the pest risk posed by such imports. It is motivated by the philosophy that it is better to endure some inconvenience and expense in an effort to exclude the exotic pests, rather than submit to losses involved following their entry and establishment.

Various kinds of plant/ planting material is being imported into the country of which the material being imported in bulk for sowing/ planting carry the maximum risk as thorough examination and treatment becomes difficult and planting area required is also too large. Also, quarantine processing is often restricted to smaller samples derived from them and based on results of these samples the whole consignment is rejected/ detained or released after giving appropriate treatment. The bulk consignments meant for consumption pose lesser hazards. However, certain small samples meant for research purposes especially germplasm or wild relatives or landraces of a crop are more likely to carry diverse biotypes/ races/ strains of the pest and are of immense quarantine importance. Besides, in case of true seed, generally, risks are more due to deep-seated infections than with surface-borne

contamination. However, import of vegetative propagules present a much higher order of risk than true seeds.

There are several examples of transboundary movement of pests along with plants and planting material which at times have lead to epidemic situations; viz., Late blight of potato (*Phytophthora infestans*) introduction into Ireland from Central America in mid nineteenth century which resulted in complete devastation of potato crop; Chestnut blight (*Endothea parasitica*) was introduced into the USA around 1904 along with nursery stocks from the Orient; Coffee rust (*Hemileia vastatrix*) suddenly appeared in 1982, there was hardly any healthy coffee plant in the whole of Sri Lanka; Onion smut (*Urocysti scepulae*) got introduced into Switzerland from France in 1924; cotton production in USA was drastically reduced due to the introduction of cotton boll weevil (*Anthonomus grandis*) around 1892 from Mexico; the wine industry of France was almost wiped out due to introduction of exotic pests viz., grape root aphid (*Phylloxera vitifolia*) in the middle of 19th century, downy mildew (*Plasmopara viticola)* and powdery mildew (*Uncinula necator)* in quick succession from America and; Stem and bulb nematode (*Ditylenchus dipsaci*), a seed borne nematode, has spread to most European countries with the movement of infested seeds of alfalfa and bulbs of flowering plants.

India also has its share of devastations through introduction of exotic pests along with imported planting materials causing serious crop losses from time to time. These included: Late blight of potato (*Phytophthora infestans*) in 1883; Coffee rust (*Hemileia vastatrix*) in 1876; Flag smut of wheat (*Urocystis tritici*) in 1906; San Jose scale (*Quadraspidiotus perniciosus*) in 1930; Fluted scale (*Icerya purchasi*) in 1912; Codling moth (*Cydiapomonella*) in 1919; Coffee berry borer (*Hypothenemus hampei*) in 1990; Potato wart disease (*Synchytrium endobioticum)* in 1973; Potato cyst nematodes (*Globodera pallida, G. rostochiensis*) in 1960's; Apple scab (*Venturiaina equalis*) in 1975 ; *Banana bunchy top virus* and *Banana mosaic virus* in 1943; *Lantana camara* in 1809. However, the most damaging loss in the world history was the Great Bengal Famine of 1942, when more than 10 million people died due to starvation and it is the largest known human loss due to a plant disease world over; was mainly attributed due to the leaf spot/ blight caused by *Helminthosporium oryzae*. After 1995, with the liberalization of trade under World Trade Organization (WTO), the quarantine set-up including legislation and infrastructure of the country has been reviewed. As far as legislation is concerned, the Destructive Insects and Pests Act was legislated by the British government ruling the undivided India in 1914 which covered three nations of the present day world- India, Pakistan and Bangladesh. After partition, India retained the DIP Act, 1914 under its original name revising it as per requirements over the years through various amendments. Recently, after the WTO came into force,India legislated the Plant Quarantine (Regulation of Import into India) Orderin 2003, henceforth referred to as the PQ Order, which came into force from April 1, 2004 (http://plantquarantineindia.nic.in/PQISPub/pdffiles/pqorder2015.pdf).

However, the risks are not fully realized by many and this has led to circumvention of quarantine procedures and policies developed as safeguards. Circumvention of safeguards implies a negative image of plant quarantine believed to be too conservative and/or plant introduction specialists or plant breeders being too liberal towards risks associated with the entry of germplasm. This chapter discusses the procedures and issues related to plant quarantine in relation to international exchange/ introduction of planting material into India.

Health Testing of Plants/ Planting material under Exchange

The National Bureau of Plant Genetic Resources (ICAR-NBPGR) is the nodal organization in India to undertake quarantine processing of all planting material of germplasm (true seed as well as vegetative propagules) including transgenic planting material meant for research purposes both for public and private sector. Each year NBPGR on an average receive more than 70,000 samples in the form of germplasm and trial material for quarantine clearance.

Sampling: Health testing is usually carried out with a small portion of seed drawn from the seed lots. The objective of sampling is to ensure that the portion of the seed taken for testing is a true representative of the entire lot. However, in case of quarantine for small samples of germplasm, the entire sample is examined and tested by non-destructive methods. But if the detection technique is destructive, a good sampling procedure is adopted. Also sampling is essential while dealing with bulk consignments to get a uniform and representative sample for testing from plant quarantine viewpoint.

Methodology for Quarantine Processing:

Joint inspection: A consignment containing sample(s) is opened and samples counted and verified with the accompanying papers from the exporting country including presence/ absence of required certificates (Import Permit, Phytosanitary Certificate, etc). Samples are then processed by a team of experts from Pathology, Entomology and Nematology disciplines. The Quarantine processing involves examination of material under exchange by naked eye or with the help of magnifying glass (visual inspection) for detecting the presence of insect damaged seeds, dead or actively moving larval and adult stages, flour, webbing, presence of excreta, soil clods, plant debris, discoloured, deformed, malformed seeds, bunt balls/ spores, ergot sclerotia, rust pustules/ spores, crust of downy mildew/ spores, weed seeds, presence of yellow discolouration around the hilum, nematode galls, etc.

Specialized detection of pests

Samples are thereafter processed through various specialized techniques for each discipline. On detection of a pest the course of action is decided depending upon the type/ category of pest detected.

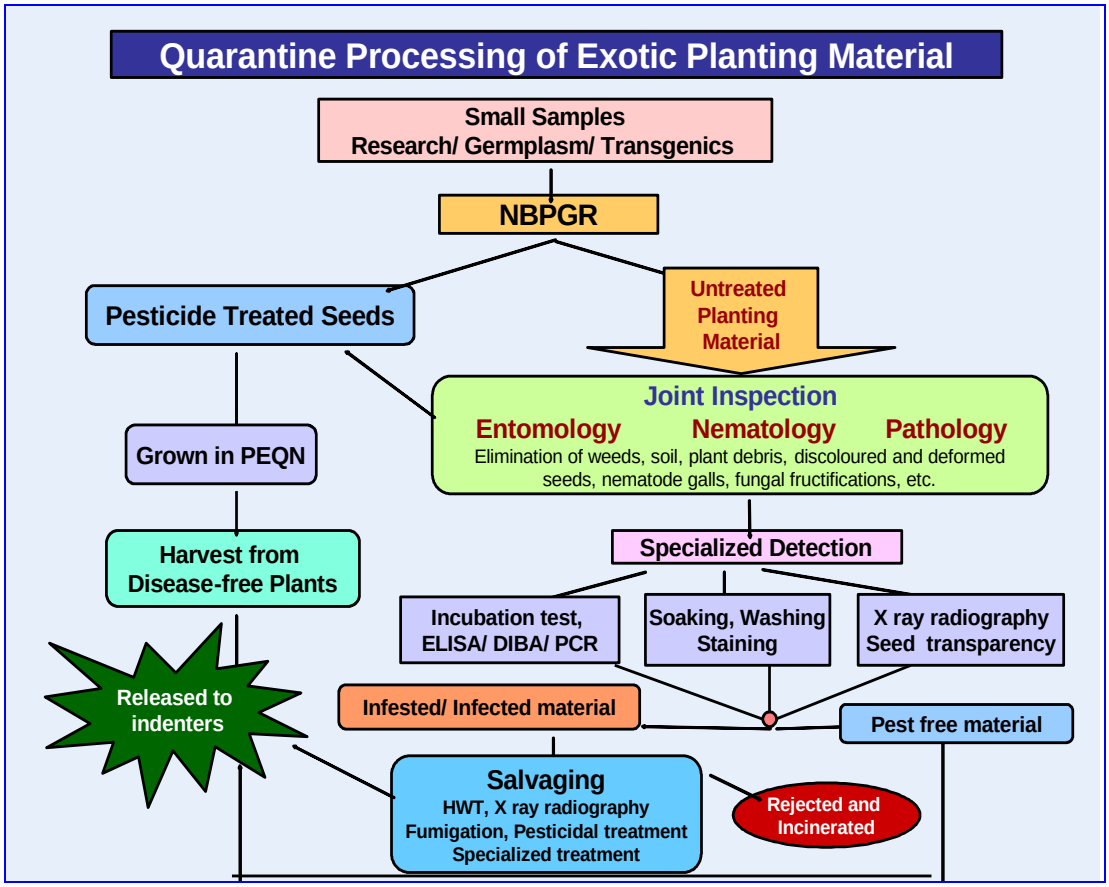

Figure 1: Flowchart of Quarantine Processing of Exotic Planting Material

Fungal and bacterial pathogens

- Examination of suspension after seed washing is used for detecting surface borne pathogens. The seeds are shaken in water and the resultant suspension is examined for spores of smuts, bunts, downy mildew, powdery mildew and some of the fungi like *Protomyces macrospores* under the compound microscope.
- Blotter method is used for detection of many fungal and bacterial pathogens capable ofproducing mycelial growth and fruiting structures under the incubation.Seeds are placed on moist filter paper in plastic petriplates and incubated at 20±1°C under fluorescent tubes in alternating cycles of 12 hours light/ darkness for 7 days show growth of fungi and bacteria.

Seed-borne viruses

- Seeds known or suspected to carry seed-borne viruses are grown in insect proof post-entry quarantine net house/ environment controlled screen houses. Seedlings showing viral symptoms are uprooted and burned. Produce from only healthy plants is released to the indentors.
- Infectivity test is done to assaythe presence of virus by inoculating leaf extracts of

seedlings showing symptoms on indicator hosts. This method reveals the symptom-less or latent infections of plants not observed in grow-out tests.

- Observation of sample from leaf showing viral symptoms under the transmission electron microscope also reveals the size and shape of the virus particles, if present.
- For serological diagnosis of plant viruses enzyme-linked immunosorbent assay (ELISA), a relatively simple, rapid and sensitive technique is used for simultaneous testing of a large number of samples.

Insects and mites

- X-ray radiography, used to detect seeds infested with phytophagous chalcidoids, bruchids and certain other insect groups that do not exhibit any external symptoms on seed surface. Based on literature survey and past experience a list of >340 plant genera has been drawn up (Bhalla *et al*., 2002), that are compulsorily subjected to X-ray radiography. On developing the X-ray plates, insects if present, are hand-picked and healthy seeds released to the indentor.
- Transparency method is used for detecting infestation in small seeds and seeds of family Graminae. The seeds are boiled in lacto-phenol solution (phenol, lactic acid, distilled water and glycerin in the ratio of 2:2:2:1 respectively) for 1-2 hours depending on the hardness of the seeds. This renders them transparent to reveal insect infestation.

Plant parasitic nematodes

- Soaking of seeds known/ suspected to carry seed-borne nematodes in water overnight softens the seeds which are teased/ crushed enabling the nematodes, if present, to come out in water.
- Soakingof some plant material in water and when sieved through nematological sieves (the finest sieve is of 400 mesh per linear inch) reveals nematodes that are retained on the sieve. These are recovered and examined under the compound microscope for identification.
- Staining techniqueis used for quick detection of nematodes in vegetative propagules where a part of the plant tissue (especially roots) is boiled in acid fuchsin lacto-phenol solution for a few minutes and de-stained in clear lacto-phenol. The nematodes, if present, retain the red stain more deeply than the plant tissue and can easily be detected under stereo microscope.
- Examination of accompanying soilshows the presence of viable nematodes especially ectoparasites and cysts of cyst forming nematodes.

Disinfection/ disinfestation of material: The material is salvaged to the extent possible using physical, chemical, physico-chemical treatments including Hot Water Treatment

(HWT). Germplasm of pulses is grown in Green House/ Screen House for virus indexing. Material found chemically treated with pesticides is grown in Post-Entry Quarantine Nursery (PEQN) for expression of disease symptoms. Only harvest from disease free material is released to the indenter. A flow chart of different steps involved in quarantine processing of exotic germplasm at NBPGR is given in Figure 1.

Few details of the various methods used for disinfection/ disinfestation are:

Mechanical cleaning: The soil clods, plant debris, weeds, discoloured, deformed and shrivelled seeds are mechanically cleaned by hand picking. The vegetative propagules are cleaned by excising the infected portion.

Hot-water treatment (HWT): for this treatment various temperature and time combinations are used for eliminating pathogenslike fungi, bacteria and nematodes. The treatment is given in hot water treatment tank fitted with heaters of different capacities, stirrer, thermostat and contact thermometer for controlling the water temperature.

X-ray radiography: is used to separate insect infested seeds (which do not have any external symptoms) from healthy ones. On developing the film exposed to X-rays, the infested seeds can be easily distinguished and are hand-picked from the seed geometry. In case of real-time X ray machines, the process is much faster and salvaging is done immediately after the image of infested sample appears on the computer screen.

Fumigation: it is one of the most effective methods used in quarantine for eliminating insects, mites and nematodes done either at atmospheric pressure or under vacuum conditions.Atmospheric fumigation is done at normal air pressure in an air tight container using Ethylene dichloride - carbon tetrachloride (EDCT) mixture (3: 1) @ 320 mg/l at 30°C for 48 hrs.Vacuum fumigation is done in especially designed fumigation chamber which helps in hastening the penetration of the fumigant through tightly packed material or internal infestation. The commonly used fumigants are ethylene oxide and carbon dioxide mixture, hydrogen cyanide gas and methyl bromide.

Pesticidal treatment: it is the most practical method to use in quarantine for effective control of surface feeding insects and mite, nematodes, etc. Few nematodes in rooted plants, cuttings, tubers and other vegetatively propagated plant material, dipping in systemic chemicals at various concentration x time has been found to be effective.Chemical seed dressing is generally given for eliminating seed-borne fungi and bacteria. Various systemic fungicides are available which are used as seed dressing or dips for vegetative propagules.

Spirit wash: is used for eliminating the seed borne rust spores of safflower rust (*Pucciniacarthamii*). Contaminated seeds are taken in a test tube containing ethyl alcohol (spirit) and a pinch of river sand and stirred with a mechanical stirrer for 30 seconds. The spores adhering to the seed surface get dislodged and separate out in the alcohol.

Acid wash: with concentrated sulphuric acid is used for destroying the spores of sugarbeet rust (*Uromycesbetae*) adhering to sugarbeet seeds. The contaminated seeds are stirred with a glass rod in the acid for one minute. The treated seeds are immediately washed under the running tap water to remove traces of acid and are sun dried.

PEQ isolation growing: Chemically treated seed materials are grown in isolation in post-entry quarantine isolation for one crop season for the detection of seed borne pathogens. Healthy seeds from the uninfected plants are then released to the indentors.

Tissue culture technique is the safest for eliminating associated pests from the germplasm, wherever possible. Shoot tip culture is an efficient technique of raising pathogen free plants from infected germplasm. This method is based on the possibility of obtaining pathogen free progeny from a systematically infected plant by meristem culture. The technology is particularly helpful in eliminating viruses from valuable germplasm.

Prophylactic treatments: All the imported paddy samples are given prophylactic HWT at 52^{o}C for 30 minutes against seed borne fungi, nematodes and bacteria. All trial material are also given mandatory fumigation with suitable fumigant; and vegetative propagules are given prophylactic pesticidal dip/spray treatment depending on the nature of material before release.

For import of germplasm, import permit and phytosanitary certificateis essential. NBPGR, under the PQOrder 2003, has been authorized to issue import permit and handle quarantine processing of germplasm material including transgenics and for issue of phytosanitary certificate. It is well equipped with most modern quarantine facilities including transmission electron microscopeand a containment facility (Containment Level-4) for quarantine processing of transgenic germplasm in a risk-free manner (Mathur and Lal, 1996, Khetarpal *et al.*, 2006a). NBPGR also has a well-equipped quarantine station at Hyderabad, which mainly deals with the export samples of International Crop Research Institute for Semi-arid Tropics.

Research institutions of public and private sector interested in importing plants or planting material should request NBPGR for Import Permit (IP) which is not transferable. On arrival, the quarantine scientists carefully process the material. In case material is found to be infected/ infested with pests, all efforts are made to salvage the material. Only in rare cases, when the material cannot be salvaged it is incinerated (Figure 1). In case post-entry quarantine examination of the imported material is required, it is done at quarantine greenhouse facility, at ICAR-NBPGR, New Delhi and its Regional Station, Hyderabad.

Quarantine Procedures for Import of Planting Material

Pre-entry Plant Quarantine Requirements

(a) Import Permit is a statutory requirement from the country of import and the conditions/ additional declarations laid on it need to be fulfilled by the country of

export.

(b) Phytosanitary Certificate is also a statutory requirement and is a proof that the consignment has been examined according to the requirements of the importing country and found to be free from the quarantine pests.

(c) PRA carried out by the country of import for which desired information is supplied by the country of export.

(d) Approval of Post-entry Quarantine (PEQ) Growing Facility is essential as all planting material to be subjected to PEQ inspection as per the PQ Order 2003.

Post-entry Quarantine Requirements

(a) Screening at NBPGR - the examination is carried out to make sure that phytosanitary conditions laid down in the import permit have been taken care of and material is free from exotic pests.

(b) Examination of the material after entry, PEQ growing and treatment if required.

Over the years, during quarantine processing, a large number of pests have been intercepted in imported germplasm and other research material (Table 1). The intercepted pests can be divided into different categories:

(i) Pests not known to occur in India

(ii) Pests with different races/ biotypes/ strains not known to occur in India

(iii) Pests intercepted on a new host or are from a country from where they have never been reported before

(iv) New pest species hitherto unreported in science

(v) Pests reported to be present in India but with a wide host range

These interceptions, especially of pests and their variability not yet reported from India [Category (i) and (ii)] signify the importance of quarantine in preventing the introduction of destructive exotic pests.The categories (iii) and (iv) pests are not expected in the sample as per the PRA which is literature-based and since no records are available on the pest/ host their presence is unexpected and hence, important from quarantine view point. The last category (v) - pests with a wide host range are critical andcould become invasive in case they find suitable biotic and abiotic environment (Khetarpal and Gupta, 2008).

The importance of quarantine has increased manifold in the WTO regime and adopting not only the appropriate technique but also the right strategy for pest detection and diagnosis would go a long way in ensuring pest-free exchange of germplasm and trade, and is considered the best strategy for managing transboundary movement of pests. It is clear that under the present international scenario, the plant protection specialists have a major role to play not only in promoting and facilitating the export and import in the interest of their

respective nations but also in protecting the environment from the onslaughts of invasive alien species. Since, quick and reliable diagnostics of pests is crucial for safe exchange of plant/ planting material, the workers need to improve their knowledge and skills for precise diagnosis. Therefore, training is required for quarantine officials, germplasm curators and scientists who are involved in assessing the conformity to the international standards.

Table 1: Few important quarantine pests intercepted in exotic germplasm (Not yet reported from India)

Pest	Host	Source
Insects		
Acanthoscelides obtectus	*Cajanus cajan*	Brazil, Colombia
	Phaseolus vulgaris	Italy, Nigeria
Anthonomus grandis	*Gossypium* sp., *Hibiscus* sp.	USA
Bruchus ervi	*Lens culinaris*	Several countries
Bruchus dentipes	*Vicia faba*	Syria
Bruchophagus gibbus	*Trifolium*spp.	USA
Ephestia elutella	*Triticum aestivum*	Italy
Oscinella frit	*Hordeum vulgare*	Sweden
Pachymerus lacerdae	*Orbygnyapha lerata*	Brazil
Nematodes		
Ditylenchus dipsaci	*Allium cepa*	UK
Heterodera schachtii	*Beta vulgaris*	Germany
Pratylenchus hamatus	*Mentha spicata*	Brazil
Pathogens		
Fusarium nivale	Cereals	Germany, Hungary, Italy, Mexico, Sweden, UK
Peronospora manshurica	*Glycine* spp.	Several countries
Uromyces betae	*Beta vulgaris*	Belgium, Germany, Italy, UK, USA
Alfalfa mosaic virus	*Vigna unguiculata* *Glycine max*	Nigeria Taiwan
Broad bean stain virus	*Vicia faba*	Bulgaria
Cowpea mottle virus	*Vigna unguiculata*	Philippines
Pea seed-borne mosaic virus	*Vicia faba*	Bulgaria
Tomato black ring virus	*Vigna unguiculata*	Nigeria
Weeds		
Cichorium spinosum	*Trifolium alexandrium*	Egypt
Vicia villosa	*Medicago lupulina*	Switzerland

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9

Plant Genetic Resources - Conservation and Management

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Plant genetic resources are one of tremendous practical significance for supporting life on earth to generating wealth of nations and therefore conserving these resources is a mean of safeguarding the life exploited by agriculture industry, forestry and aquaculture to supply food, feed medicines, fibre, fuel and many industrial products. Conservation ensures that future generations will benefit from these genetic resources. The basic objective of plant genetic resources conservation is the protection of genetic diversity at intra-specific and inter-specific levels to ensure the future adaptability of cultivars and wild populations, to preserve data and traits that ensure sustainable agriculture; to promote the use of genetic resources in commerce and biotechnology; to conserve diversity for cultural reasons.

The Genebanks -a part of *ex-situ* conservation strategy, are under increasing scrutiny as *in-situ* strategies are now gaining more importance mainly due to climatic changes. Therefore, there is an urgent need for precaution in terms of effectiveness and economy in genebank operations with respect to species diversity and responsibilities when it comes to connect it with farming community. The design and management of genebanks must be done keeping in view the conditions, availability of human and financial resources, and the low-cost methodologies available in the country concerned.

The National Genebank at NBPGR has the mandate to

- Undertake and promote long-term conservation of plant genetic resources in genebank and assist in the *in situ* conservation efforts.
- Act as the repository of notified /released varieties/cultivars, parental lines of released hybrids, genetic stocks, as also regional repository of duplicate collections of specific crops as a part of the global system.
- Monitoring and maintenance of the extant collections, facilitating the organization of regeneration programme.
- Complimenting the activities of National Active Germplasm Sites (NAGS) to ensure availability of exotic and indigenous germplasm through periodic seed increase for evaluation, utilization and conservation.
- Developing and operating a database and information network system on PGR.
- Conducting research related to medium and long-term conservation of germplasm.

- Support/assist in human resource development by organizing short/medium-term training courses on PGR conservation activities at national, regional and international levels.

Genebank activities

The major activities at any genebank involves

i. Acquisition of germplasm-either through explorations or by introduction/exchange with other institutions within country or outside
ii. Processing of the received germplasm for conservation involving seed health/ quarantine testing, viability testing and moisture estimation and its storage in genebank
iii. Allotment of National Identity once the germplasm is qualified for conservation at genebank.
iv. Regular monitoring of the conserved germplasm in storage and regeneration whenever/ wherever required.
v. Distribution of the germplasm to the users
vi. Value addition to conserved germplasm through characterization/ preliminary evaluation, if not available for effective utilization
vii. Documentation of the passport information of the conserved germplasm

Conservation procedures

The principles governing conservation procedures are maintenance of viability, vigour and genetic integrity of the germplasm. Desiccation tolerant seeds which maintain high viability under storage at low temperatures are called as orthodox seeds and at National Genebank seeds of all agri-horticultural crops are conserved at -18°C to -20°C. Seed viability and vigour is controlled by genetic, biochemical and physiological characteristics but to a large extent influenced by storage temperature and humidity. Thus at NGB seed lots with initial high viability (above 85%), free of diseases, pest or pathogen are conserved to minimize the loss of vigour and viability during storage. Thus the various procedures adopted for successful conservation of any germplasm are:

i. Testing of initial moisture content as soon as the germplasm is received (using ISTA 2005 guidelines).
ii. Drying of the received germplasm in controlled conditions (drying rooms/cabinets maintained at 15°C and 15% RH)
iii. Seed viability testing using ISTA prescribed optimum conditions for various crops
iv. If there is a occurrence of dormancy then evincing dormancy breaking protocols
v. Packaging and vacuum sealing the samples in trilayer aluminum foil pouches
vi. Documentation and labeling of the packets and then assigning location in LTS module
vii. Monitoring of the conserved germplasm (for ten years in most of the crops except oilseeds where after 5-6 years the monitoring is done)

viii. Regeneration of germplasm, in case of loss of viability or less seed quantity but frequency of regeneration is kept minimum to avoid risks of genetic shift, drift and contamination which are compounded with each regeneration cycle (upto a maximum of two or three cycles)

Standards for germplasm processing

Standards for seed storage

- Type of collections: Depending upon the objectives and storage period the collections are classified as
- Active Collection*:* These are stored for medium period of 2-10 years. They are being regularly used for research and distribution to bonafied users.
- Base Collections: These are conserved for long duration, 15 years or more for safety and posterity. They are to be used only in emergency, such as loss of an accession, for regeneration of *active collections* and to support other complementary conservation activities.

Seed storage conditions

- For Base Collections- -20°C with seed moisture 3-7%; the standards should not be relaxed
- For Active Collections- Conditions that can maintain seed viability above 65 per cent for 10-20 years. However, decrease in seed moisture content is *cost effective* than temperature.

Storage modules

- **Short Term:** Temperature 15-20°C, and 30-40 per cent RH for seed processing.
- **Medium Term:** Temperature 0-10°C and 25-30 per cent RH. Adequate to maintain seed viability for 2-10 years, suitable for *active collections*.
- **Long-Term:** Temperature -20°C and no control of RH. Adequate to maintain seed viability for 10-50 years, suitable for *base collections.* The seeds are kept in hermetically sealed containers with around 3-5% seed moisture.

Seed drying procedures

- Seeds should be dried soon on receipt to avoid further deterioration
- Avoid damage for seed during drying
- Lower the seed moisture for prolonging longevity using dhimmified chambers (using desiccant for drying at 10-25°C and 10-15 per cent RH.

Seed cleaning and health

The seed to be stored should be free from weeds, pest and diseases. Crop specific knowledge is preferred. Germplasm received at National Genebank is subjected to visual and microscopic examination to remove weed seeds, soil particles, broken parts of flower, debris or any inert material before sending to quarantine examination for any seed borne pathogens and pests.

Storage containers

Containers should be moisture proof and amenable to hermetic sealing. Test to ensure non-exchange of moisture. The containers available are:

- Plastic containers
- Aluminum containers
- Multi-layered pouches of polyethylene, Aluminum etc.

Accession size

In *base collections* minimum size should be sufficient to meet the requirement of one regeneration cycle and test of viability. 2000 viable seeds for homogenous crops and 4000 seeds for heterogeneous crops (it may be decreased for difficult to multiply crops). In *active collection*, as large quantity as possible to avoid frequent regeneration.

Monitoring of seed viability

- Seed accessions are monitored in genebanks to adjudge the minimum acceptable limits of germination (65-85%)
- Assessed by means of a germination test
- A minimum of 100-200 seeds are used for germination test at the start of seed storage.
- Period between viability monitoring vary among species and storage conditions. For *base collection* 10 years or as required, for *active collections* 5 years
- For viability monitoring randomly drawn 50-100 seeds are used
- Well equipped laboratory and laboratory equipment's for viability tests, in some cases specialized equipment's, such as X-ray machine etc. are required
- Based on situation, seedlings raised during germination testing *be* used for regeneration of an accession

Regeneration standards

Regeneration standards are needed to ensure the seed viability does not fall below acceptable limits.

- Seeds for *base collection* should be pathogen free and of highest possible quality. Regeneration should be undertaken when viability falls (85±10%)

- Plant 100 or more seeds for regeneration to avoid large losses of alleles
- Seeds used for regeneration should be genetically as close as possible to the original sample

Standards for information management

Data as complete as possible both on *base* and *active collections* along with standards descriptor as prepared by IPGRI and International centers (Passport information proforma for breeding lines/variety- Annexure 1):

- Passport
- Management
- Characterization
- Evaluation
- Mode of reproduction

Standards for germplasm exchange

While supplying the seeds to bonafied user it is recommended that

- Seeds be supplied in suitable containers
- Adequate information should accompany for effective use
- Seed lot should have high viability level
- Quarantine regulations are strictly followed
- Should accompany MTA as per national requirement

Genebank personnel and training

Trained staff with regular updating of skill is essential.

Standards for maintenance of modules

- A voltage stabilizer to protect from fluctuations in electric supply
- A built-in electrical control panel providing complete operation information
- Indicator lights to display operating condition
- ***Spares:*** compressor unit, thermostat, fan motor, expansion valves, compressor contractor overload relays, and fuses etc. should always be kept for immediate replacement.

Standards for safety and security of modules

Every effort must be made to ensure utmost safety of equipments. Following need attention:

- Uninterrupted power supply
- Fire precautions

- Security
- Refrigeration standards and equipment used should be as per DSSF
- Construction and installation-following guidance given by DSSF
- Safety of personnel: protective clothing, etc.

PASSPORT INFORMATION PROFORMA FOR BREEDING LINES/VARIETY GERMPLASM CONSERVATION DIVISION

ICAR-National Bureau of Plant Genetic Resources New Delhi

Supply/co-operating institute:
Date:

Developer:
Collaborating Institute: Name of Scientist(s) and Address:-

Conserved at: ICAR-NBPGR, Pusa campus, New Delhi

S. No	Crop	Bot. Name	Crop-group	Country of Origin	Donor institute	Other ID	Pedigree	Source	Bio-Status	state	Novelty/ Unique features		

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Plant Tissue Culture Interventions for Management of Plant Genetic Resources

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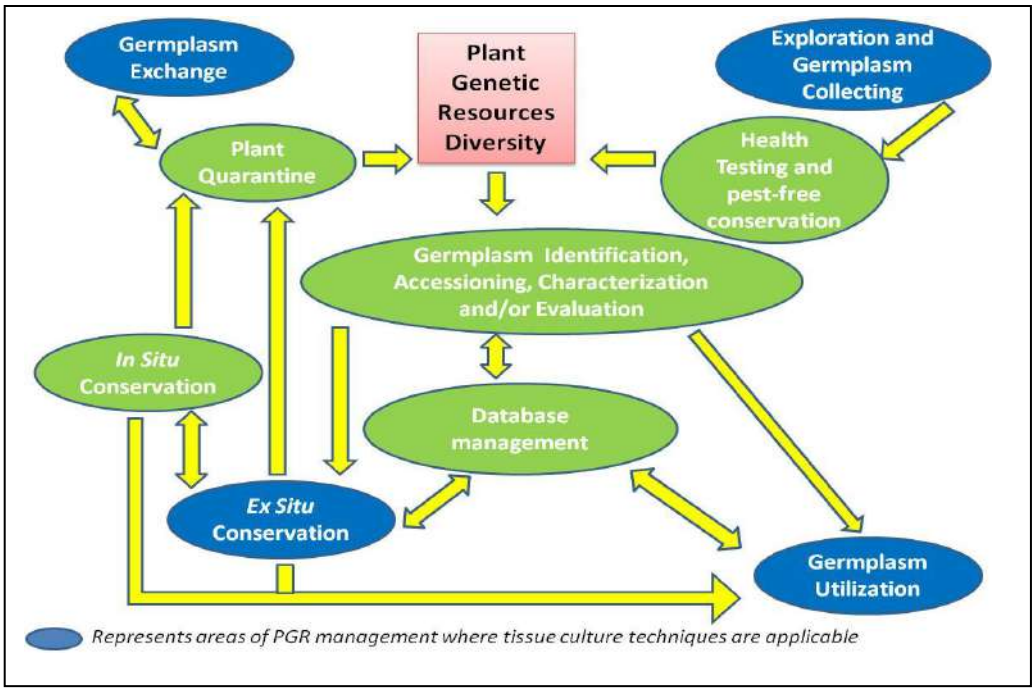
Plant tissue culture (PTC) is a classical biotechnological technique which involves culturing plant seeds, organs, tissues, cells, or protoplasts on a chemically defined synthetic nutrient media under aseptic and controlled conditions of temperature, light, and humidity. The whole science and industry of PTC is based on the concept of 'cellular totipotency', wherein each plant cell has the inherent potential give rise to all cell types (including a whole plant), under suitable conditions. The other important trait is 'plasticity'; the ability of plants to alter their metabolism; growth and development to best suit their environment. Thus, PTC is a valuable tool for research on morphogenesis, cell signaling, physiology and molecular biology, as well as for crop improvement. Today, it is being widely used for different types of applications such as (i) meristem culture for propagation of virus-free plants;(ii) tissue and organ culture for micropropagation, induction of genetic variability; (iii)protoplast and cell suspension cultures for transgenic plants/genetic engineering and secondary metabolite production;(iv) somatic embryogenesis for clonal multiplication and synthetic seeds; (v) embryo culture for seed dormancy breaking and wide hybridization rescue; (iv)anther or pollen culture for producing haploid plants. This chapter provides an overview on how PTC techniques are being effectively used for various aspects of management of PGR like collecting, exchange, disease eradication, utilization and most importantly for conservation of germplasm (Figure 1).

PTC in *Ex Situ* Conservation of PGR

Seeds (orthodox type) are the most predominant form of PGR conservation under *ex situ* conditions, in gene banks. However, this method cannot be adopted for many crops due to biology of the plants - such as highly recalcitrant nature of the seeds (*e.g.* tropical fruit trees), clonal nature of valuable varieties (*e.g.* many root and tuber crops), or species normally do not produce seeds (*e.g.* banana, garlic) and threatened plants were obtaining adequate number of seeds, as per gene bank standards, is rather difficult. In such species/crops alternative conservation strategies are used, including the conservation in field gene banks or the storage of plant material *in vitro,* using PTC. *In vitro* conservation refers to maintenance of germplasm in a relatively stable form under more or less defined artificial environment and nutrient conditions in *In vitro* Gene banks (IVG). Vegetatively/clonally propagated crops are



essentially conserved in their true-to-type forms through *in vitro* conservation(short-and medium-term storage) and cryopreservation(for long-term storage).Since cell and callus cultures are more prone to somaclonal variations, shoot-tips, meristems, nodal sections, dormant buds *etc.*, are the most preferred explants for *in vitro* and cryo-conservation of the clonally propagated crops. Cryostored and regenerated cultures are routinely tested for their genetic fidelity, especially at the time of protocol development. The flow of germplasm for *in vitro* conservation (medium-term) in the *in vitro* Active Genebank (IVAG) and *in vitro* cryopreservation (long-term) in the *in vitro* Base Genebank (IVBG) is graphically depicted in Figure 2. The step-wise procedures and requirements for application of PTC for IVAG and IVBG are enumerated hereunder.

In vitro Culture Initiation and Multiplication

Germplasm acquisition for conservation

- The germplasm for in IVG should be obtained from an authentic source (e.g. field genebank, greenhouse, glasshouse, net house, or other IVG) along with its correct taxonomic identity and passport information. Characterization and/or evaluation data are desirable.

- Samples for IVG may be obtained as whole plants, vegetative propagules, *in vitro* cultures and in exceptional cases as seeds/embryos (e.g. threatened species, crop wild relatives).

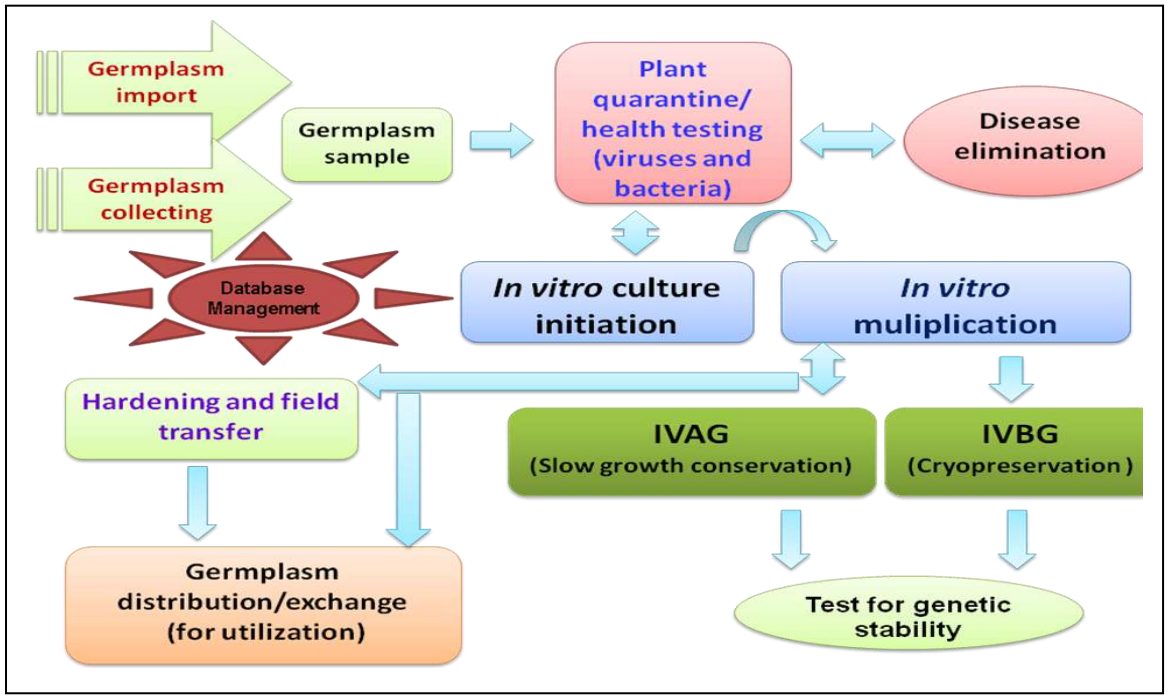


Figure 2: Major operations involved in the establishment of *in vitro* Gene Banks (IVGB)
IVAG = *In vitro* active genebank, IVBG = *In vitro* base genebank
(*Source:* Agrawal *et al.*, 2013)

Quarantine/ health testing of samples

- Germplasm samples should ideally be sourced from disease-free mother plants/propagules.
- Exotic collections should be accompanied with suitable Phytosanitary Certificate from the source country, and have undergone suitable quarantine checks in the host country.
- In case of indigenous collections obtained as live plants/cuttings, they should be suitably planted in screen house or in growth chamber for establishment and growth.
- Prior to *in vitro* introduction, disease status, especially endogenous bacteria and viruses should be checked in the samples, as per available guidelines.

Establishment of in vitro cultures

- Depending on the species, suitable explants need to be selected for its introduction in tissue culture. These may be meritems, shoot tips, nodal cuttings, young shoots derived from whole plants or vegetative propagules like budwood, suckers, tubers, bulbs, corms and rhizomes; in rare cases like triploid/seed-sterile plants, floral buds may also be used.

- Type, source and physiological stage of explant are the important factors in optimizing the tissue culture protocol.
- The explants must be disinfected to remove microbial contaminants on the surface. using one or more disinfectants, like ethanol (70%), sodium/calcium hypochlorite, commercial bleach/ chlorine water and antibiotics, in appropriate concentration. Pre-soaking in an anti-oxidant solution (e.g. ascorbic acid) may be useful for woody plants. Explants drawn from field need to be checked for presence of mites, thrips and other insects.
- Using sterilized forceps, explants are transferred into a sterile container and rinsed 2-3 times with sterile distilled water. Edges of the explant are cut with the help of sterile blade.
- Prior to culture initiation, base of the explants should be streaked on a medium (mostly nutrient agar) for testing any microbial contaminant.
- With sterile forceps, explants are planted on a suitable culture medium (e.g. Murashige and Skoog). Each tube should be covered with a plastic cap and sealed with parafilm/cling film; in some cases, cotton plugs give better growth.
- Culture tubes are transferred to a growth chamber (temperature 25±2^{o}C) under suitable photoperiod and light intensity, depending species.
- The crop/genotype specific media should be pre-determined, as also the additives such as charcoal, plant growth regulators, antioxidants *etc.* for desired response from explants. Culture initiation media should be gelled with a clear gelling agent (e.g. gellan gum rather than agar) for easier detection of bacteria and other contaminants.
- Bacteria-free cultures should be indexed for viruses using standard serological (ELISA), molecular (PCR based) and ultrastructural (electron microscopy) techniques; virus-free material should preferably be used for subsequent steps.

In vitro multiplication

- Only aseptic cultures, established *in vitro*, should be selected for multiplication and generating adequate number of explants for subsequent IVAG conservation.
- Shoots should be removed aseptically under a laminar air flow and micro-cuttings of apical and/or nodal sections be transferred to a new medium. Individual test-tube should be labelled clearly using a standardized format either manually or using a data logger barcode/QR code.
- The medium required for culture initiation may be different from that required for shoot multiplication, plantlet regeneration and storage.
- Cultures must be transferred to a regulated culture room at the appropriate temperature/light regime. Periodically cultures should be monitored for

contamination, hyperhydricity, growth abnormalities (somaclonal variation, loss of regenerability etc.) after each subculture cycle.

In vitro Conservation Using Slow-Growth Strategies

Cultures for medium-term conservation in IVAG are normally subjected to a suitable slow-growth storage strategy to save time, energy, costs and space. Slow growth strategies are based on extending subculture duration, without risking germplasm loss and compromising genetic stability through stressful treatments. Stored cultures need to be scored for viability, chlorosis, defoliation, browning, tip necrosis, hyperhydricity and contamination. Depending on the method applied, cultures can remain viable from 6-30 months, without subculture.

Growth retardation by changing physical parameters

- Decreasing temperature at which cultures are maintained (e.g. from 25˚C to 4-15˚C). It is the most commonly used and single method for restricting the growth of tissues, found suitable for temperate crops, as tropical species are cold-sensitive and may exhibit chilling injury.
- Reducing light intensity at which cultures are maintained been found beneficial for reducing growth in several species, especially temperate crops.
- Changing the culture vessel enclosures influences the rate of evaporation of water content of the medium. Use of polypropylene caps instead of cotton plugs as has been found to significantly increase storage period of the cultures.
- Size and type of the culture vessels also plays a very important role e.g. sterile, heat sealable polypropylene bags and large glass bottles have proven to be better than glass test tubes.
- Minimal growth can be achieved by lowering the available oxygen level to tissues. The simplest method is to cover the tissues with mineral oil layer. However, some associated problems with decreased oxygen concentration may be encountered (e.g. vitrification, partial or complete necrosis), which depends on species/genotypes.

Growth retardation by chemical changes in medium

- Decreasing the nutrient contents in the culture medium and/or sucrose restricts the growth of cultures, thereby increasing the subculture duration.
- Use of osmotic regulators like mannitol and sorbitol (3-6% w/w) minimizes growth by imposing osmotic stress on the cultures.
- Use of inhibitory level of growth retardants (2-50 mg/l) such as maleic hydrazide, abscicic acid, n-dimethyl succinamic acid, cycocel and phosphone-D to the culture medium leads to slow growth. However, use of growth retardants is generally not preferred due to its effects on genetic integrity of the cultures.

Induction of in vitro storage organs

- This method is useful for crops with natural storage organs, such as alliums, ginger, turmeric, taro, yam, potato, sweet potato, etc.
- Inclusion of high sucrose (6-8% or more) in the medium in combination with light/dark conditions is conducive for *in vitro* organ formation (micro-corms, bulbs, rhizomes, tubers etc.), which prolongs the storage life of cultures up to 1-3 years under *in vitro* conditions.

Monitoring Genetic Stability of In Vitro Conserved Germplasm

In vitro selection pressure can potentially generate variants or mutants. Also some genotypes have a propensity for producing off-types and variants (due to natural chimeras). Maintaining shoot and plantlet regeneration through preformed (original) meristems (apical and axillary buds) and avoiding adventitious shoots greatly aids the maintenance of trueness-to-type and genetic stability and offset the risks of SCV. However, some slow growth treatments can aggravate abnormal morphogenetic responses. Thus, *in vitro* conserved cultures should be periodically monitored for genetic stability by suitable methods (phenotypic, chromosomal, cytological, epigenetic and genetic molecular tests, RAPD, DNA methylation, RFLP, AFLP, SSR, etc.

IVBG - Long-Term Conservation Using Cryopreservation

Cryopreservation is the technique utilized for long-term storage of living cell, tissues, organs and other biological resources at ultra-low temperatures (−196°C) usually in liquid nitrogen (LN).Under such a low temperature, the metabolic activity of the tissue virtually comes to a halt, and thus, it can be conserved without significant change in its viability. This technique provides safe, cost-effective conservation of non orthodox seeds, vegetatively propagated species and biotechnologically important plant cell lines. Tissue from the field that are amenable to cryopreservation include seeds, embryos, embryonic axes, budwood and pollen. In vitro derived tissues normally cryopreserved are meristems, shoot tips, nodal cuttings, cell suspensions and somatic embryos. Cryopreservation can be attempted using classical (freeze induced in which cooling is performed in the presence of ice) or new cryopreservation (vitrification-based in which cooling normally takes place without ice formation) techniques, as detailed hereunder.

Classical cryopreservation techniques

This method follows protocols which induces a freeze-dehydration process using as low freezing regime. Ice is initially formed in the extra cellular solution, during slow temperature decrease and this external crystallization promotes the efflux of water from the cytoplasm and vacuoles to the outside of the cells.Classical cryo-techniques have been successfully applied to cell suspensions and calluses of different plant species, apices of cold-tolerant plants as

well as shoot-apices from tropical species. Following successive steps need to be defined for each species.

- Selection of material and pre-treatment of donor plants**:** Generally, rapidly growing meristems which are small, relatively resistant to freezing, possessing fewer or smaller vacuoled cells and dense cytoplasm are preferred for cryopreservation.
- Pre-treatment of isolated explants: It refers to the period in culture before cryopreservation begins. Following the aseptic excision of the explants and culture on high sucrose medium (0.1-0.5 M) the plant tissues are cold-hardened by using effective pre-growth additives such as mannitol, sorbitol, proline or dimethylsulphoxide (DMSO) in pre-growth culture medium.
- Application of cryoprotectant: Just before freezing cryoprotectants are applied (individually or in combination) which include DMSO, glycerol, proline, sucrose, sorbitol and polyethylene glycol (PEG). Cryoprotectants must be screened for toxicity without freezing, using concentration and time as variables for the investigation.
- Freezing, storage and thawing: Slow freezing is carried out by using controlled freezing apparatus, which help in extra-cellular ice formation. The frozen specimen is then transferred to storage in LN. Specimens are thawed rapidly or slowly to avoid the risk of damage to cellular integrity due to recrystallization of any residual ice formed during freezing.
- Recovery growth: Usually recovery growth is carried out on a defined medium supplemented with activated charcoal to adsorb toxins released by damaged cells and growth substances to stimulate the desired responses. It is sometimes necessary to avoid the osmotic shock and photo-oxidation during regrowth phase.

New cryopreservation techniques

These are vitrification-based procedures that involve cell dehydration prior to cooling by exposure of samples to highly concentrated cryoprotectant solution (usually plant vitrification solutions, PVS) and/or by air desiccation. Physical process of vitrification *perse* is defined as the transition of the liquid phase to an amorphous glassy solid at the glass transition (Tg) temperature. When the explants undergo desiccation, rapid or ultra-rapid cooling and thawing process, the glass transition may help in preventing tissue collapse, solute concentration and pH alterations. Different techniques of cryopreservation are given in Table 1.

PTC in Germplasm Exchange and Quarantine

Exchange of PGR for research, conservation and basic plant breeding purposes, is channeled and regulated through government agencies to facilitate safe movement of germplasm i.e. avoidance or minimization of risks of introducing unwanted pests, pathogens, weeds etc. Failure to follow these procedures can result in the introduction of new pests into countries or regions, and can ultimately result in epidemics that threaten food security and biodiversity.

Table 1: Techniques of Cryopreservation

	Technique	Explants	Procedure	Remarks
1.	**Pregrowth**	• Meristems • Shoot tips/buds • Somatic embryos	Tissue/explants are cultured in presence of cryoprotectants (DMSO, PEG, Sucrose etc.) for few days/weeks followed by rapid freezing in LN	Applicable to very limited species and response is genotype dependant, e.g. *Musa*, sweet potato
2.	**Pregrowth-desiccation**	• Shoot tips/buds • Nodal cuttings • Somatic embryo cultures • Zygotic embryos	Tissue/explants are cultured in presence of cryoprotectants (DMSO, PEG, Sucrose etc.) for few days/weeks followed by air/silica gel desiccation and rapid freezing in LN	Applicable to very limited species (asparagus, oil palm, melon, rapeseed, coconut)
3.	**Encapsulation-dehydration***	• Meristems • Shoot tips/buds • Nodal cuttings • Somatic embryos • Zygotic/microspore embryos • Cell suspensions	Explants are encapsulated in sodium/calcium alginate The beads precultured with high sucrose (0.3 to 1.2 M) for 1-7 days. Beads desiccated to 13-35 % moisture content in air or silica gel, followed by rapid freezing in LN	Although several parameters need to be standardized, has been successfully been in many clonally propagated crops
4.	**Vitrification**	• Meristems • Shoot tips/buds • Nodal cuttings • Somatic embryos • Zygotic embryos • Cell suspensions	Tissue/explants may be pretreated (cold /simulated acclimation.) followed by loading, cryoprotection, rapid freezing in LN, and deloading	Protocol has been applied to a wide range of culture types and plant species
5.	**Droplet vitrification**	• As above	Same as vitrification, expect freezing is carried out directly in LN at ultra rapid rates, by exposing the explants directly to LN, placed on aluminum foil strips	Protocol has been applied to a wide range of culture types and plant species with higher success rate than vitrification
6.	**Encapsulation-vitrification**	• As above	A combination of techniques 3 and 4. Encapsulated explant is dehydrated with cryoprotectant solutions. Rapid freezing in LN followed by rapid thawing and deloading	Used in a few plant systems
7.	**V-Cryo-plate and D-Cryoplate vitrification**	Meristems Shoot tips/buds Nodal cuttings Somatic embryos	Similar to techniques 5 and 6, except use of aluminum cryoplates (which have wells, dia 1.5 mm, depth 0.75 mm).	A relatively new technique successfully applied in large number of species

(*Source: Agrawal et al., 2013*)

Advantages of germplasm exchange using tissue cultures

In vitro plantlets offer a safe method for germplasm exchange of valuable material, especially in case of vegetatively propagated crops. PTC simplify the quarantine procedures for exchange of germplasm due to safe phytosanitary status as well as easy transport and following reasons:

- The technology of meristem culture alone or in combination with thermotherapy in vegetatively propagated crops can be utilized for virus and viroids elimination.
- *In vitro* exchange is preferable for highly perishable propagules of vegetative crops which are difficult to transport over long distances such as cuttings, budwoods, suckers, tubers.
- Bulky explants/material *viz.*, bulbs, grafts and seedlings which need excessive care during transport, can easily be shipped as tissue cultured plantlets in larger quantities.
- Using *in vitro* methods, test tubes can be conveniently packed in a dispatch-box and sent via air freight. *In vitro* cultures can withstand rigors of transport and survive at ambient temperatures (15-30^{o}C) for 3-4 weeks.
- PTC assures the availability of true-to-type clones round the year thus, the germplasm is readily available for the purpose of distribution at the time of the request.

Practical difficulties encountered

- Delay at airport/receiving stations due to administrative reasons such as lack of necessary documents (phytosanitary certificate etc.).
- Dislodging or melting of agar due to mishandling during transit and high temperature. This causes leakage of medium in certain containers thereby resulting in contamination or necrosis of cultures.
- Desiccation of cultures due to delay in transport or high temperatures, loss of regeneration capacity of the cultures and misidentification of cultures due to loss of labels.

PTC for Germplasm Collecting

In vitro collecting (IVC)is a technique that involves directly placing materials into sterile tubes in the field, for transport to a distant laboratory. It is a supplemental tool for obtaining plant tissues of both crop and wild species for *in vitro* propagation and conservation. IVC comprises a support activity and is not merely for propagation.

Explants and Methods of IVC

Explants having short viability or longevity *viz.*, dormant buds, shoots, shoot tips, etc. or having excessive volume (bulky) and weight (large fruits, whole plant etc.) can be easily

collected through IVC as it offers non-invasive explant isolation and safe collection of live tissues. Examples include coconut, banana, rubber, cocoa, rare and wild species. The steps followed are:

- Selecting the appropriate tissue for inoculation (zygotic embryos or vegetative tissues, like budwoods, shoots, apices or leaves).
- Cutting the tissue to an appropriate size, eliminating soil residues and visible diseases and pests by a preliminary wash.
- Surface sterilizing the tissue with fungicides and antibiotics, followed by washing to remove the disinfectant and cutting off unnecessary or damaged tissue.
- Inoculating into culture vessels containing a nutrient medium and closing the vessels.
- Transferring the material to incubation chambers or sent for transport.

Factors affecting IVC

- Tissue size and type, collection of disease free explants/tissue, surface-sterilization of plant tissue, nutrient medium.
- Success of IVC depends on the quick processing of the explants in the field and correct procedures followed in the lab for elimination of any type of infection.
- Limitations include high skill requirement, melting of medium during transport, desiccation of cultures and misidentification of cultures due to loss of labels.

PTC for Disease Eradication

Meristem Culture and Cryotherapy for Virus-free Germplasm

Shoot tip or meristem culture is used since many decades to eliminate viruses from vegetatively propagated plants. The meristematic zone is usually free from viruses due to its fast cell multiplication rate and absence of vascular connections. Isolation and regeneration of tiny meristems results in production of virus free plants. Since regeneration ability of the explants is directly proportional to the size of the shoot tip, but pathogen eradication is more efficient using small shoot tips (0.2-0.4mm), pathogen eradication with meristem culture is challenging as it is very difficult to isolate small meristem mechanically and regenerate a whole plant.

Cryotherapy has emerged as a novel technique for elimination of systemic plant pathogens, such as viruses, phytoplasmas and bacteria, by treating shoot tips with LN using cryopreservation protocols.Cells of the meristematic zone are smaller as compared to more differentiated cells and have smaller vacuoles and higher nucleo-cytoplasmic ratio and hence more likely to withstand dehydration as compared to differentiated cells. Cryotherapy takes advantage of these differences in physiological status of differentially localized cells by destryoing infected differentiating cells by a breif cryo-treatment in LN. The uneven

localization of viruses and obligate vasculature-limited microbes in shoot tips allow selimination of the infected cells by injuring them with the cryo-treatment and regeneration of only healthy shoots (free from pathogen).Cryotherapy therefore, has much higher potential to regenerate pathogen-free plantlets as compared to traditional methods like meristem culture, chemo-and thermo-therapy.

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11

Principles and Applications of Cryopreservation

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Cryopreservation, or freeze-preservation, is the storage of biological materials at ultra-low temperatures (¯180ºC to ¯196ºC), created by using liquid nitrogen. Cryopreservation reduces cell and tissue deterioration by virtually halting metabolism and, hence, ensures theoretically 'infinite' period of storage without any loss in viability or vigour, or any changes in genetic makeup. Based on the needs and the institution, the priority for germplasm conservation at the National Cryogenebank, ICAR-National Bureau of Plant Genetic Resources (ICAR-NBPGR) has been assigned. The Cryogenebank has the following specific need based priorities for diverse germplasm and explants from both sexually and asexually propagated species for effective management. The germplasm priorities followed at ICAR-NBPGR are:

- Species producing intermediate & recalcitrant seeds, with sizable indigenous diversity
- Vegetatively propagated species
- Threatened and endangered plant species with critically small population size
- Wild and weedy relatives of crop plants
- Registered germplasm
- Exotic germplasm
- Medicinal and aromatic plants
- Core collections
- Released varieties

Explants for cryopreservation

Following explants are generally processed for cryopreservation of various categories of germplasm:

1. Seeds
2. Embryos and embryonic axes
3. Pollen grains
4. Dormant buds
5. Meristems and shoot apices
6. Genomic resources

Cryopreservation procedures and standards are being refined on a species-specific basis as soon as the new reports on post-storage behaviour and cryoprotocols become available. Good quality pest free germplasm is preferred for best results. In case of samples stored without being checked for infections, they require a screening and disinfection before plating/ field sowing or *in vitro* culturing on retrieval from storage. There are no 'standard procedures' for conservation of the germplasm of non-orthodox-seeded species. The curator and genebank managers need to investigate the post-harvest biology of the seeds in question, and thus ascertaining the best procedures for new accessions about which little or nothing has been reported.

With proven wide applicability of cryotechniques in plant sciences, diverse germplasm of national and international importance is being cryoconserved at National Cryogenebank at ICAR-NBPGR. Over a span of 32 years cryoprotocols have been developed, in most cases on species-specific basis, using explants like seeds, embryos, embryonic axes, pollen and dormant buds of these 'difficult-to-store species'. In view of storage capacity of National Cryogenebank at ICAR-NBPGR to hold quarter million samples (in 1 ml capacity cryovials), presently stored in about 42,000 containers (cryovials of 2, 5, 50 ml capacity, and polyolefin tubings of 2 different diameters) holding more than 14,000 accessions belonging to more than 820 plant species. Cryopreservation protocols are being standardized primarily for the tropical species of horticultural, plantation, agro-forestry and industrial importance. Desiccation-freezing, vitrification and encapsulation-dehydration and classical techniques of step-wise slow freezing are being experimented and applied on large scale to range of germplasm to establish base collection of minor tropical and temperate fruits, *Citrus* species, neem, black pepper, mulberry and mango in various forms.

Processing of various explants for cryopreservation

Following steps are to be followed for processing and cryobanking of various explants:

1. Handling and laboratory storage of material (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
9. Retesting of cryostored material after stipulated periods.

Requirements for processing and cryobanking of various explants

Handling and storage of material

Propagules or explants collected or procured for cryoreservation should be carefully handled and stored at optimum temperature (10-20^{o}C) in a refrigerator till processed for cryopreservation. The intermediate and recalcitrant seeds loose viability sooner and their fruits/ seeds should be stored at 5-6^{o}C temperature if chilling tolerant and at 15-18^{o}C if chilling sensitive before and during processing to ensure a high viability percentage. Such seeds may be stored in sawdust, charcoal or after treating with fungicides like Bavistin or Thiram powder. This practice will ensure high survival and pathogen free laboratory storage with retention of initial moisture content.

Collection and handling of pollen grains in viable condition is a primary requirement for any experimental study on pollen. Generally, pollen collected soon after anther dehiscence gives optimal response. For collection of pollen grains in viable conditions butter paper sheets, needles, muslin cloth, open trays, forceps, filter assembly, filter paper sheets, etc. are required.

For non-orthodox species whole fruits of consistent maturity are collected from the parent plants to ensure high quality and are transported to the lab by courier enclosed in moisture-retaining bags or containers. Extracted seeds are used up for experimentation within few hours to few days. Such seeds are stored in sawdust, charcoal or after treating with fungicides like Bavistin or Thiram powder at temperatures between 15 to 20^{o}C. This ensures high survival and pathogen free laboratory storage with retention of initial moisture content. Seed morphology is studied to decide explants- whole seed/embryo/ embryonic axes most appropriate for conservation and for ascertaining the ability of explants to regenerate. Moisture content is determined and the most suitable desiccation method is devised. The sensitivity to desiccation of seeds, embryos and embryonic axes can be evaluated by assessing the relation between the moisture content and the germinability of explants. The moisture content at which survival of seeds, embryos and embryonic axes is highest is the critical moisture content of that explant.

Extraction and excision of explants

Extraction of seeds from fruits should be carefully taken up especially in case of intermediate and recalcitrant seeds. In case of large seed excision of embryonal axes or for vegetative tissue, meristems, shoot apices and dormant buds should be extracted initially under the stereo microscope. Morphology of the embryonal axes, shoot apices, meristematic tissues and dormant buds should be studied in advance for quick and complete excision of explant for various experimentations.

For extraction and excision of seeds from fruits, embryonal axes, meristems, dormant buds, etc. a laboratory should be equipped with knifes, sieve, fine forceps, scalpel handles, surgical blades, syringes and needles.

Estimation of moisture content

Moisture content can be measured using low constant temperature oven method described earlier. The weight of the sample container should be comparable to the explant to be weighed. In case of very small explants like embryonal axes, meristems and pollen grains, small vials made of aluminum foil can be used instead of glass weighing bottles.

For moisture content estimation, a laboratory should be equipped with a hot-air oven, glass weighing bottles and a precise electronic balance along with accessories like pestle and mortar and blades for crushing/slicing of the explant material.

Germination and viability measurements

In a cryolab, all the incoming accessions should be checked to ensure high viability percentages and at least 90% of the explants in a sample destined for storage should germinate. Requirements and methods for germination and viability testing would depend on the explant being processed. In many tropical species seed germination methods are not readily available leading to detailed experimentations on standardizing the method.

For viability testing of embryonal axes, embryos, shoot apices, meristems and dormant buds *in vitro* culturing is to be undertaken. Most of the studies are usually carried out using MS basal medium.

In vitro germination is the most commonly used technique in pollen physiology. Compositions of these pollen culture media are as follows:

	Brewbaker and Kwack's Medium	**Robert's medium**
Sucrose	10%	20%
Boric acid	100 mg l^{-1}	10 mg l^{-1}
Calcium nitrate	300 mg l^{-1}	-
Magnesium sulfate	200 mg l^{-1}	-
Potassium nitrate	100 mg l^{-1}	100 mg l^{-1}
Calcium chloride	-	362 mg l^{-1}
Tris	-	60-130 mg l^{-1}

Seed germination testing will require germination media like brown towel sheets/ germination paper/ petriplates lined with filter paper/ agar solidified in petriplates. Further, the seeds should be incubated in a seed germinator or BOD (----expand it) where temperature, humidity and light conditions are controlled. A cryopreservation lab dealing with intermediate and recalcitrant seeds should be equipped with the facilities of a tissue culture lab (for details see chapter on *in vitro* conservation) or should work in collaboration

with nearest tissue culture laboratory. To test the germination of pollen grains germination media, microslides, cover glass, microscope and humidity chamber under desired temperature are required.

Standardization of desiccation protocols

Rate of desiccation of explant is a very critical step in cryoprocessing of germplasm. To achieve different dehydration rates and depending upon the type of material following methods can be used:

a. Air drying - Whole seeds
b. Silica gel drying- Whole seeds, embryos, embryonic axes, dormant buds, pollen
c. Laminar air flow drying- Embryos, embryonic axes, shoot tips, meristems and dormant buds.
d. Flash drying- Embryo, embryonic axes
e. Chemical dehydration (Cryoprotectants) - Embryonic axes, shoot tips, meristems, dormant buds, pollen.

Desiccation of seeds, dormant buds and pollen with higher moisture content is usually carried out on charged silica contained in air tight desiccators. Regular monitoring of the sample should be done to ensure a high viability and low moisture content. Excessive desiccation can lead to loss in seed viability.

The embryonal axes usually have very high moisture content as they are excised from sterilized seeds, which are washed 3-4 times with sterilized distilled water. Desiccation of embryonal axes can be achieved by air desiccation in laminar air flow or through use of cryoprotectants (vitrification) or encapsulation-dehydration, depending upon the species and the methodology followed. The apical and axillary meristems can also be used as explant for cryostorage of many species. They are also dehydrated using vitrification or encapsulation-dehydration methods.

It is essential to have a strategy to examine the effect of different parameters on survival when a new system is handled for cryopreservation. It may be essential to develop new methods or optimize standard protocols for specific plant species or tissue types. For this, optimal factors for additives and procedures like pregrowth, dehydration, cryoprotection, vitrification, encapsulation and recovery growth would need to be worked out.

For desiccation of orthodox seeds, the seed sample is tied in muslin cloth, labeled properly and then kept in silica for drying. Air desiccation of embryonal axes is carried out in laminar airflow cabinet while for vitrification, autopipettes, disposable pipette tips, a vial stand, sterile filter papers, sterile petriplates and cryoprotectants like DMSO (Dimethyl sulfoxide), ethylene glycol and glycerol are required. For encapsulation, alginic acid (sodium salt),

calcium chloride and an orbital shaker with or without temperature and light control with 80-100 rpm speed are required. For fast desiccation, flash drier (customized equipment) is used.

Standardization of freezing protocol

The rate of freezing plays an important role in successful cryopreservation of tissues since it affects the amount and rate of formation and size of ice crystals, as well as the injury due to solution effects that occur during freezing. Slow freezing permits the efflux of cellular water and facilitates extracellular freezing as a consequence of the imposed reduction in temperature. The resultant protective dehydration is attained at temperatures of about -30 to –40^{o}C and is governed by the cooling rate, the type of cryoprotectant and the permeability of the membrane to water. Different types of explants may require different cooling rates, however, a uniform cooling rate of 0.3 to 1^{o}C min^{-1} from ambient temperature is effective for a wide variety of explants. For freezing, only two options are available *viz* rapid freezing by direct plunging in liquid nitrogen or slow freezing using a programmable freezing device.

Slow freezing has been in use since last 20 years and has led to the successful cryopreservation of several temperate fruit species. The protocol involves pregrowth of samples on medium with cryoprotectants including DMSO or non-penetrating chemicals followed by slow dehydration in a cryoprotectant solution at intermediate concentration. The freezing rate is usually less than one degree per minute from 0°C to the terminal prefreezing temperature of ⁻35°C to ⁻40°C, followed by a rapid immersion in LN. Crystallization occurs in the intercellular spaces and the cytoplasm probably vitrifies. Rewarming of the samples should be rapid.

Storage of explants in cryotanks

All types of explants are to be finally packed in airtight containers before cryostorage. Glass containers can shatter when warmed from sub-zero temperatures, so polypropylene screwcap cryovials are generally used. Polypropylene cryovials of different sizes (1ml, 2ml, 5ml, 50ml) designed for low temperature works are commercially available. Pollen desiccated to suitable moisture content can be stored in aluminum packets, gelatin capsules or polypropylene cryovials. For rapid rates of cooling, cell suspensions are placed in stainless steel or silver hypodermic tubing or glass capillaries. The size of the cryovial to be used will depend upon the size as well as the quantity of the seeds/ embryonal axes/ pollen grains to be stored. Large seeds can be stored in heat-sealable polyolefin tubing with cork stoppers or in goblets in sleeves.

There are no guidelines regarding the minimum number of seeds to be cryostored per accession. For explants like embryos, embryonic axes, meristems, shoot tips and pollen, there is no standard recommendation for the minimum number of explants to be stored. It usually depends upon the availability of the material, percentage survival and on the plan of retesting.

Successful cryopreservation depends upon the use of good equipment and an efficient inventory system. A good liquid nitrogen storage tank should be self contained, vacuum insulated vessel with the LN (liquid nitrogen) reservoir and samples in the same cavity. A consistent temperature will thus be maintained by this. Storage of germplasm in vapour above the LN is preferred by most workers since it is relatively safe for working personnel.

Maintenance of cryostored germplasm

Liquid nitrogen boils off continuously as heat infiltrates into the cryotanks through the sidewalls and the access port on the top. Additional loss of LN occurs when warm samples are placed in the tank. Each tank has a static holding time, which is the maximum time for which a tank can hold a particular quantity of LN, after which more LN has to be replenished. This is dependent on the rate of evaporation and the capacity of the tank. Evaporation rate of LN for most of the tanks is 0.5 to 1.5% of its capacity per day and accordingly, replenishment of LN is required about two times per week to maintain the temperature of the cryotank between $^{-}160$ to $^{-}180^{o}$C.

Retesting of viability

Post-cryopreservation handling procedures and regeneration protocols need also be fully standardized and readily available to ensure high survival rates. It is essential to monitor the viability of explants after regular intervals to ensure that no deterioration is there over the time. For thawing of samples, a 37-39^{o}C water bath is required.

Requirements for establishing a cryolaboratory

For starting a small-scale cryolaboratory narrow mouthed cryocontainers having capacity to hold 11-60 liters of liquid nitrogen can be used. Further, after experimentation, the samples, which are to be stored for long-term, should be transported to the nearest large-scale cryolaboratory. For transportation, cryo-dry shippers of different capacities available from various international companies can be used, wherein liquid nitrogen is adsorbed by the filled-in porous material, thus reducing the risk of accidental spillage of liquid nitrogen during transportation. The laboratory should be fully equipped to handle diverse type of plant tissues and requirements for application of all the new techniques. Conventional thermometers cannot be used for monitoring of temperature during slow freezing thus a copper-constant thermocouple capable of recording temperature upto $^{-}200$°C should also be available for accurate measuring. A constant and reliable supply of liquid nitrogen is essential. For a small-scale laboratory, one can install a LN production plant or a supplier may be hired who can regularly supply LN. For a large-scale laboratory where LN consumption is high, a vertical LN station / horizontal reservoir of extra large capacity (4000-8000 l) can be maintained near the cryobank which should be constantly filled to ensure continuous supply of LN to storage tanks. In addition to the equipments mentioned above both types of cryolaboratories should be equipped with safety gadgets like gloves, aprons, face masks, face shields or goggles, etc. Other accessories like cryomarkers, glass markers,

metallic (aluminum) holdings like canes on which the cryovials can be mounted before dipping in liquid nitrogen and heavy-duty trolleys for transportation of tanks should also be available.

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Characterization and Evaluation of Plant Genetic Resources

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Characterization, evaluation and regeneration of germplasm are an important component of Plant Genetic Resources management. Characterization of germplasm primarily describes the basic characters of an accession whereas evaluation of germplasm reveals the actual value of the accession. The characterization of germplasm deals with the understanding and recording of highly heritable characters which may be used in establishing taxonomic identity, while, the germplasm evaluation deals with assessing the agronomic potential of an accession including quality parameters and response to various abiotic and biotic stresses. Thus, evaluation is the key to accelerate utilization in crop improvement programme. Maintenance of germplasm without losing genetic integrity is also an important activity of PGR management. It covers the whole range of activities starting from the receipt of the new samples by the curator and growing them for seed increase, characterization and preliminary evaluation, and also for further detailed evaluation and documentation.

Characterization is defined as assessment of attributes of a given accession that are relatively stable across environments. This is done usually for qualitative, monogenically inherited, environmentally stable characters (colour, pubescence and shape of plant parts) which are easy to measure and are used for classification and categorization of accessions. Evaluation is the measurement of attributes for given accession and mainly deals with often quantitative, polygenically inherited and environmentally sensitive traits (yield attributes, days to flowering and maturity, nutritional and phytochemical contents, tolerance to abiotic and biotic stresses) which are relatively difficult to measure.

Characterization

Characterization provides a standardised record of readily assessable plant characters which, together with passport data, go a long way to identify an accession (Frankel, 1986). Characterization descriptors include spike/panicle shape, seed shape and colour, and other characters which are generally more of taxonomic type. Their recording along with the passport data provides an overall picture of the range of diversity in the collections. There is different techniques used for characterization depending upon the crop. The most commonly used techniques for characterization of PGR is morphological characterization where descriptors namely stem, leaf, flower colour, etc. are recorded based on visual observation from the field experiment using suitable experimental design. Besides this biochemical characterization is done for parameters important to individual crop namely, oil per cent in

oilseed crops, etc. In recent times, molecular characterization based on DNA markers or molecular markers are also gaining importance because of the lack of environmental influence on these molecular makers. The highly reproducible molecular markers like Amplified Fragment Length Polymorphisms (AFLPs), Simple Sequence Repeats (SSRs) and Single Nucleotide Polymorphisms (SNPs) should be used for characterization.

Characterization data resulted in botanical identification and establishing diagnostic keys for distinguishing them, categorize into different groups, assess inter-relationships among accessions, and estimate the extent of variation in the germplasm collection and to identify and remove duplicates from the existing germplasm collection. Besides, another important activity seed multiplication/seed increase can also be done. It needs care as it involves the risk of losing a particular accession due to poor adaptation, pest and disease damage, introducing admixtures through contamination or error and altering the genetic composition of the original genetic makeup through conscious (human) or unconscious (natural) selection. During initial seed increase, observations on agro-morphological traits and other traits of interest are recorded. Duplicate accessions are also identified at this stage and promising ones are identified for intensive evaluation. Preliminary evaluation is defined as recording of a limited number of additional agronomic characters important to a particular crop and thought desirable by users. Most importantly, descriptors and descriptor states related to characterization are site data, and characters of leaf, stem, flower, fruit and seed.

Evaluation

Evaluation of PGR is basically systematic evaluation in order to know the potential of germplasm after augmentation of genetic resources. Evaluation includes recording of potential agronomic traits, quality traits, biotic and abiotic stresses for its use in specific purpose and in specific environments. The principal goal is to identify useful genes or genotypes for desired traits. Generally, it starts with either large number of germplasm from National Genebank or germplasm already identified from characterization and preliminary evaluation or based on passport information, and is conducted at a single adapted location for agronomic traits. The evaluation for biotic and abiotic stresses is conducted at the field in hot spot conditions i.e. where the stresses occur naturally. The infector row is included for uniform pathogen load in case of biotic stress evaluation. The promising accessions are tested in artificial conditions or agro-inoculation depending upon the biotic stress for confirmation of resistance. The quality evaluation is primarily conducted in laboratories through modern equipments. The evaluation is conducted with a suitable experimental design depending upon the objective and size of germplasm with at least three checks i.e. two national checks and one local check. The germplasm accessions are usually evaluated for two consecutive years for documentation and preparation of crop catalogue. For effective evaluation of germplasm, a close collaboration between curator and breeder is necessary in the context of breeding objective vis-a-vis evaluation programme. The evaluation requires multidisciplinary approaches and specific testing conditions. Such systematic evaluation activities, though

expensive and time consuming, are of great value. The multi-location evaluation is conducted for testing the germplasm at different agro-climatic condition for adaptability, resistance to different races of disease/different diseases and for different abiotic stresses.

In general, characterization and preliminary evaluation is done by the curator/germplasm scientists; further evaluation or detailed evaluation is mostly done by the breeders for taking additional information. However, no hard and fast rule prevails and the detailed evaluation can also be done by the curator in collaboration with breeders, pathologists, entomologists, agronomists and biochemists, etc. as per need.

The focused identification of germplasm strategy (FIGS) is a scientifically-proven tool that helps crop breeding programs identify useful traits in plant genetics more accurately and efficiently, improving on the limitations of more traditional approaches which are largely hit and miss (Fig.1). This is one of the efficient methods to explore PGR for climate change adaptive trait specific germplasm. It comprises a powerful algorithm that matches plant traits with geographic and agro-climatic information of the places where samples were collected. This allows the rapid searching of thousands of plant samples conserved in gene banks to pinpoint a number of high potential types that can meet the breeder's strategy.

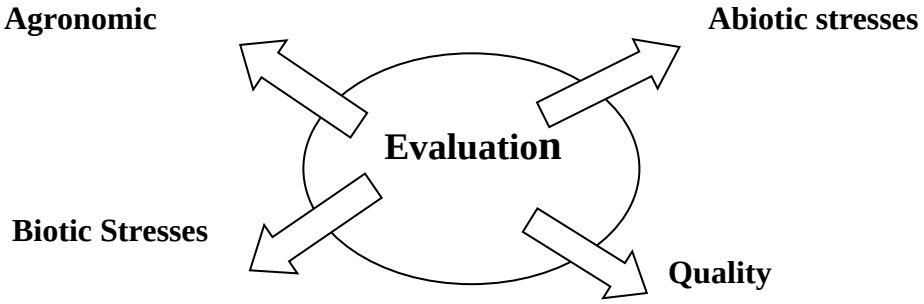

Figure 1: Focused Identification of Germplasm Strategy (FIGS)

FIGS is based on the concept that the environment strongly influences natural selection and thus affects the geographical distribution of organisms. It creates 'best-bet' trait-specific subsets of germplasm by passing accession-level information, especially agro-climatic site information, through a series of filters that increase the chances of finding the adaptive trait of interest. FIGS grew out of early work done by Michael Mackay in the 1980s, building on the core collection concept. Tasked with developing boron-tolerant wheat for Australian farmers, Mackay turned to accessions that had been collected from Mediterranean sites with soils of marine origin – soils that commonly contain toxic levels of boron. These accessions provided all the genetic variation needed to develop boron-tolerant cultivars. This helps to improve the effectiveness of crop improvement programs. The global genebanks hold more than 7.5 million accessions of crops and their wild relatives - a vital source of novel genes that can improve drought tolerance, disease resistance, and other traits. But the sheer number

of accessions makes it difficult for breeders to identify those that might have useful traits. FIGS combines agro-ecological information with data on plant traits and characteristics to narrow down the search – identifying sets of plant genotypes with a higher probability of containing specific 'target' traits.

Descriptors

Crop curators with their own experience, help of the crop advisory committee and several experts from relevant fields like biochemistry, pathology, entomology, etc. may developed list of descriptors. ICAR-NBPGR, Bioversity International and CGIAR institutions developed descriptors for characterization and evaluation of targeted crops. The use of uniform descriptors and descriptor states facilitate the utilization of germplasm by different research workers. The different kinds of descriptors are as follows:

a. Passport descriptors: recorded at the time of collection of germplasm.
b. Environmental and site descriptors: describes the environmental and site- specific parameters that are important when the characterization and evaluation trials are being held.
c. Characterization descriptors: expresses in all kind of environments.
d. Evaluation descriptors: characters used for the agronomic performance, quality parameters, biotic and abiotic stress.

In addition, the Minimal Descriptors on agri-horticultural crops published by ICAR-NBPGR are widely used for characterization and evaluation for PGR.

Core collection

Frankel (1984) termed 'Core Collection' is a subset of entire collection representing 'with minimum repetitiveness, the maximum genetic diversity of a crop species and its wild relatives. The remaining accessions of the entire collection is defined as reserve collections. It does not replace the existing collection or material from which it is obtained. Brown (1989) suggested that it should not be more than 10 per cent of the entire collection and always less than 2000 entries. They are distinct for each other genetically and ecologically. Most of the developed core collections were 5-20% of the size of the collection from which they were established. A general procedure for the selection of a core collection can be divided into five steps, which are described in the following sections:

Steps: Identify the material (collection) that will be represented » Decide on the size of the core collection » Divide the set of entire collection used into distinct groups »Decide on the number of entries per group » Choose the entries from each group that will be included in the core » validation of core collection

Function of core collection:

- Provide a reference set for comparing the novel material
- Provide set for priority handling whenever needed
- Provide appropriate set of accessions for monitoring in genebanks by routine seed testing
- Act as a priority group for safety duplication, for further distribution to regional or international genebanks or for maintenance in different conditions (e.g. as DNA libraries, in field banks or in vitro)
- Provide test material of choice for possible improved maintenance procedures (e.g. ultra-dry seeds, in vitro and cryopreservation)
- Provide benchmark standard for documentation and allow stratification of whole collection to be recorded
- Preferred material for developing authentic and accurate list of descriptors

Germplasm Field Day: Germplasm field day is the primary activity of ICAR-NBPGR where diverse germplasm is displayed with distinct traits. Breeders and crop experts are invited from all parts of country representing National Agricultural Research System (NARS) for on-spot selection of the promising germplasm of their need to accelerate the crop improvement programmes in India.

Registration of Germplasm

The Indian Council of Agricultural Research (ICAR) made ICAR-NBPGR a nodal institute to register the trait-specific germplasm developed/ identified by the researchers in India. Germplasm or Genetic stock of agricultural, horticultural and other economic crops, including agro-forestry species, spices, medicinal and aromatic plants, ornamental plants, which is unique and has potential attributes of academic, scientific or commercial value can be registered. Registered germplasm is ready material which can be utilized as donors for targeted breeding programme in India.

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Pre Breeding for Effective Use of Plant Genetic Resources

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Plant genetic resources are defined as "any material of plant origin, including reproductive and vegetative propagating material, containing functional units of heredity of plant origin of actual or potential value for food and agriculture". These resources are used, or have the potential to be used, for food and other agricultural purposes. They include crop wild relatives; other species that could interbreed with crops; wild plants that are harvested for food; landraces and farmer varieties; and formally registered crop varieties (Table 1). The diversity of plant genetic resources underpins global food security and nutrition. Plant genetic resources consist of a vast diversity of heritable traits that have enable crops to adapt to physical and biological stresses e.g. drought, heat, cold, pests and diseases. This diversity needs to be harnessed to help crop production systems adapt to the consequences of climate change.

Table 1: Types of germplasm and other genetic materials used for crop improvement

Advanced germplasm	Recently developed varieties, obsolete varieties,* and other advanced breeding materials that are created with modern breeding techniques. These usually have higher frequencies of desirable genes than landraces or wild relatives.
Landraces	Varieties that were improved by farmers over many generations without modern breeding (generally requiring extensive efforts for use in a final variety).
Wild or weedy relatives	Undomesticated plants that share a common ancestry with a crop species (sometimes very difficult to incorporate in final varieties).
Genetic stocks	Germplasm that contains defined genetic variation (i.e., mutation), sometimes incorporated into an easy-to-use genetic base, which serves as a genetic research tool
Cloned DNA sequences	Transgenic material from other organisms inserted into crops via molecular techniques. These differ from the other types of material because they cannot be used to regenerate an organism

Source: USDA\Economic Research Service, based on Day Rubenstein *et al.* (2005) and National Research Council (1993).

The sustainable use of plant genetic resources encompasses trait evaluation; pre-breeding; plant breeding, including genetic enhancement and base-broadening; diversification of crop production; development and commercialization of varieties; support to seed production and distribution; and development of new markets for local varieties and products. These

activities can contribute to addressing the impacts of climate change on sustainable crop production. Farmer varieties and landraces are generally well adapted to current conditions in their local production environments and have been a successful source for adaptive genes in crop improvement (Mba, Guimaraes and Ghosh, 2012; Lopes *et al.*, 2015). However, changing climatic conditions will mean that they may lose this adaptation (Bellon, Hodson and Hellin, 2011).

An unplanned consequence of the successes of genetic improvement is the increasingly narrow genetic base of cultivars, especially for the major crops (Tester and Langridge, 2010; Martynov and Dobrotvorskaya, 2006; Mba, 2013; Mba, Guimaraes and Ghosh, 2012; Nass and Paterniani, 2000). The increased homogeneity and uniformity (i.e. genetic vulnerability) render crops potentially more susceptible to the impact of climate change. This genetic vulnerability may be reduced by incorporating into cultivars the novel traits (e.g. resistance to biotic and abiotic stresses) that are often found in crop wild relatives (Lane and Jarvis, 2007; Dwivedi et al., 2008; Maxted et al., 2008), and landraces and farmer varieties. Pre-Breeding activities using promising landraces, wild relatives, and popular cultivars have been initiated in a diverse range of programs to overcome the significant threats to the crop productivity poses by changing climate such as rising temperatures, variable rainfall pattern and increasing diseases and pest pressures (Table 2).

Pre-breeding refers to all activities designed to identify desirable characteristics and/or genes from unadapted (exotic or semi-exotic) materials that cannot be used directly in breeding populations and to transfer these traits to an intermediate set of materials that breeders can use further in producing new varieties for farmers. The low utilization of conserved plant genetic resources in most genebanks is due to lack of documentation and inadequate description of collections, lack of the information desired by breeders, and lack of evaluation of collections.

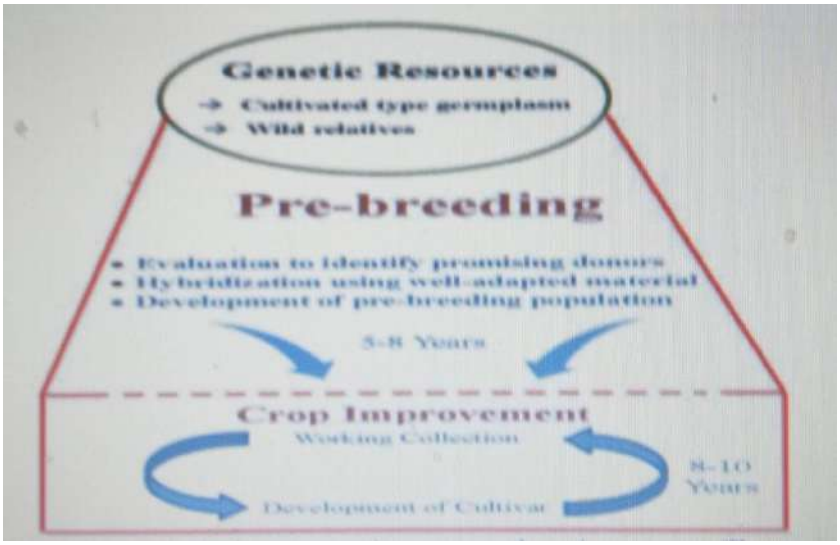

Figure 1: Pre breeding as a bridge genetic resources and crop improvement (Sharma *et al.*, 2013)

In order to fully utilize available genetic diversity in gene banks, pre-breeding or genetic enhancement of exotic/unadapted materials should be undertaken. Exotic materials include any germplasm that do not have immediate usefulness without selection for adaptation for a given area. Pre-breeding is a vital step to link conservation and use of plant genetic resources especially in breeding programs.

Pre breeding aims to reduce genetic uniformity in crops through the introduction of a wider base of diversity, as well as to increase yields, resistance to pests and diseases, and other quality traits. Pre-breeding programs can generate new base populations for breeding programs and also assist in identifying heterotic patterns for hybrid programs. Pre-breeding emphasises to provide breeders with enhanced germplasm materials which have specific traits of interest as well as a means to broaden the diversity of improved germplasm (Lokanathan *et al.*, 2003). It has been successfully used in several crops (rice, tomato, soyabean, cotton, maize, wheat, barley, groundnut, chickpea, pigeon pea, sorghum, pearl millet) by transferring the genes from wild / exotic (unadapted) species into adapted material and improved many cultivated varieties for different qualitative and quantitative traits (Plunkett *et al.*, 1987, Eshed and Zamir, 1996, Iqbal *et al.*, 2001, Sebolt *et al.*, 2000 and Seetharam, 2007).

Table 2. Traits transferred from crop wild relatives and cultivated species

Trait	Wild Relative	Cultivated species
Water stress tolerance	Slender wild oats (*Avena barbata*)	Oat (*Avena sativa*)
Leaf tolerance to cold stress	Wild grapevine species (*Vitis amurensis*)	Grape (*Vitis vivifera*)
Adaptation to high salinity and tolerance to submergence in saline water	Wild relative of rice (*Oryza coarctata*)	Rice (*Oryza sativa*)
Stress tolerance, nutritional and grain quality improvement	Wild rice (*Oryza glaberrima*)	Rice (*Oryza sativa*)
Early leaf spot resistance	Wild peanuts (*Arachis appressipila, A. paraguariensis*)	Peanut (*Arachis hypogaea*)
Drought resistance	Wild plantain (*Musa balbisiana, M. nagensium*)	Banana and plantain (*Musa acuminata, M. balbisiana*)
Adaptation to high altitudes and cool temperatures	Wild cassava (*Manihot rubricaulis*)	Cassava (*Manihot esculenta*)

Source: Brozynska, Furtado and Henry, 2015; Maxted and Kell, 2008)

The Gene Pool Concept

The crop gene pool concept developed by Harlan and de Wet (1971) is used to classify genetic relationship between crops and related taxa (Figure 2). "The gene pool is the total genetic variation in the breeding population of a species and closely related species capable of crossing with it'. The gene pool of a crop is made up of botanical varieties, landraces, inbred lines, ancient landraces, obsolete and modern cultivars, related wild species, subspecies, and weedy companion species (Haussmann *et al.*, 2004).

In crops where categorization of CWR into gene pools has not been done as per their crossability relationship, taxonomic classification can be useful. This method of classification is referred to as taxon group concept (Maxted *et al.*, 2006).

In brinjal, the primary gene pool (GP1) consists of cultivated and its wild ancestor (Figure 2) *Solanum insanum* L. (Ranil *et al.*, 2017) which can be crossed easily and produce normal fertile hybrids (Plazas *et al.*, 2016).

The secondary gene pool (GP2) includes a large number (over 40) of Solanum species that can be crossed or are phylogenetically close to brinjal, but the success of the crosses and viability of the hybrids with brinjal may be reduced. For example, some interspecific hybrids derived from GP2 are partly sterile or weak due to reproductive barriers such as with *S. dasyphyllum, S. linnaeanum Hepper* & P.-M. L. Jaeger or *S. tomentosum* L. (Rotino *et al.*, 2014 and Kouassi *et al.*, 2016).

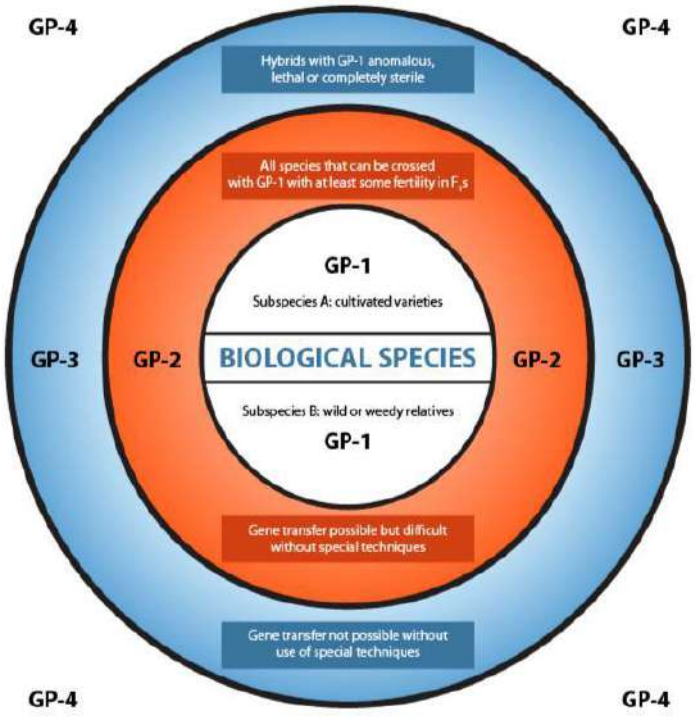

Figure 2: The modified "gene pool concept" adapted from Harlan and de Wet (1971)

The tertiary gene pool (GP3) of brinjal includes more widely related New World species which are used in its improvement programs for the transfer of resistance traits. For making successful crosses between these species, there is a need of follow specific breeding tools (e.g., *S. torvum Sw.*, *S. elaeagnifolium* Cav., and *S. sisymbriifolium* Lam.; Kouassi *et al.*, 2016; Plazas *et al.*, 2016; Syfert *et al.*, 2016).

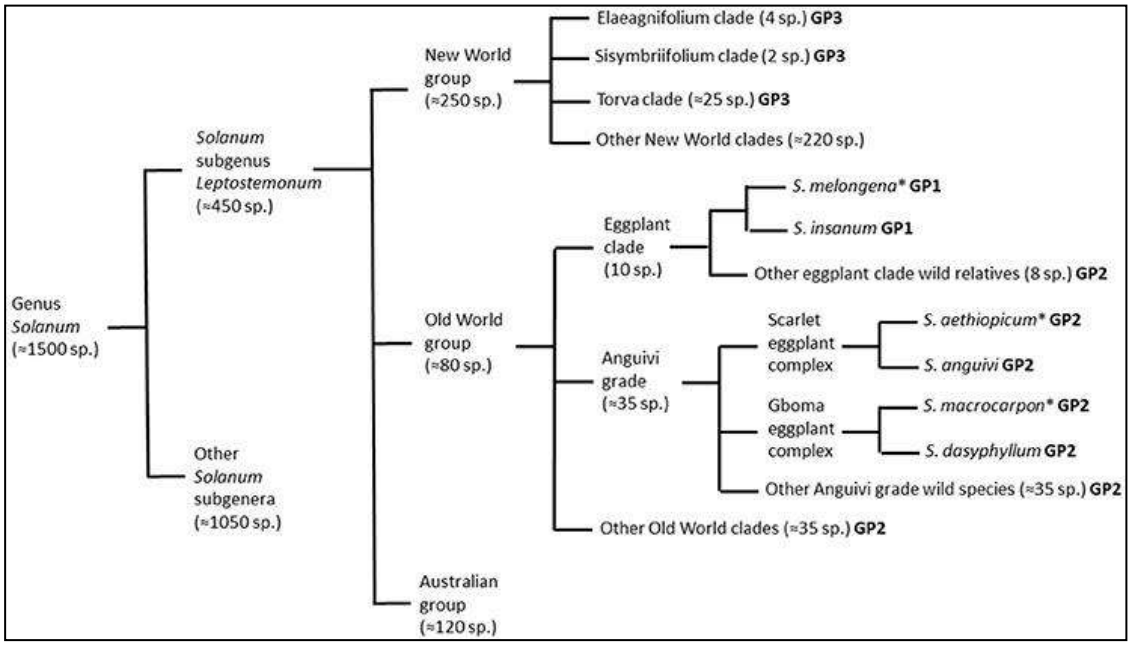

Figure 3. Schematic representation of taxonomic relationships between the cultivated brinjal eggplant (Solanum melongena) and other cultivated (scarlet eggplant, S. aethiopicum; and gboma eggplant, S. macrocarpon) and wild relatives from the genus Solanum based on Dalia et al. (2017), Nee (1999), Levin et al. (2006), Weese and Bohs (2010), Stern *et al.* (2011), Knapp *et al*. (2013), Syfert *et al.* (2016), and Vorontsova and Knapp (2016)

Genus Pisum is comprises of mainly three species i.e. *P. sativum* L. with subsp. s*ativum* (includes var. *sativum* and var. *arvense*), ssp. *elatius*, *P. fulvum* and *P. abyssinicum*. The widely used classification is given by Maxted and Ambrose, 2001 to which Vavilovia formosa was added to group four species (Smýkal *et al.*, 2011). In pea, primary gene pool consists of *Pisum sativum* including wild *Pisum sativum* ssp. *elatius*, the secondary gene pool is composed of *Pisum fulvum* and the tertiary gene pool consisting only of *Vavilovia formosa*. This is the closest species to whole tribe Fabeae that holds significant interest and breeding value in leguminous crops in the world.

Gepts and Papa (2003) modified the gene pool concept and gave an additional gene pool level known as quaternary gene pool which takes into account the biotechnological advances such as plant transformation and genomics. It harness the genes from wild species which otherwise are sexually incompatible with crop species. This type of gene pool can also contain synthetic nucleotide sequence that does not occur in nature.

Utilization of Plant Genetic Resources in Pre breeding

For the past few decades, there is a significant success in introducing different traits from wild species into cultivated crops for overcoming biotic/abiotic stresses. Latin American Maize Project (LAMP): LAMP is a real example of pre-breeding program, which includes 12 countries (Argentina, Bolivia, Brazil, Colombia, Chile, U.S., Guatemala, Mexico, Paraguay, Peru, Uruguay and Venezuela). LAMP evaluated 15,000 accessions in the first stage, with close cooperation of the public and private sectors. The great genetic variability in Latin American maize was recognized, although there is much to be known, especially its potential and its significance for breeding approaches. Thus, maize breeders now have access to the most promising stocks identified by LAMP to expand the genetic base in maize.

Recurrent Introgressive Population Enrichment (RIPE): RIPE was first adopted in 1990 by D.E. Falk in barley involving male sterile facilitated recurrent selection. The system consisted of one set of three hierarchical levels which like corn HOPE, was open-ended in that germplasm could move upward through the hierarchy and introductions could be added at the low level. However, the system was redesigned to intensify introgression at successive levels.

There are several problems that are associated with genetic enhancement programmes particularly when genes are introgressed from wild species. Some problems are listed below:

- Cross incompatibility in inter-specific crosses.
- Stability barriers and chromosome pairing in hybrids have restricted the access to genes from wild species into cultivated ones.
- Linkage drag.
- Hybrid inviability and sterility.
- Small sample size of inter-specific hybrid population.
- Restricted genetic recombination in the hybrid population.
- Lack of availability of donors for specific traits viz., resistance to diseases and Insect and pests

Introduction of late blight resistance from the wild potato *Solanum demissum* Lindl is the main landmark. The primary approach for crop improvement today remains recurrent selection among elite modern varieties. Plant breeders are continuously looking into wild species as sources of novel genes to widen the genetic base of crops (Cooper *et al.,* 2001; Hodgkin and Hajjar, 2007; Moore, 2015).

There are examples of wild gene incorporation reaching the released cultivar stage were found in soybean, pigeon pea, sorghum, phaseolus beans and cowpea. New cultivars incorporating genes from wild relatives were also reported to be in the pipeline. Over 60 crop wild species were identified to have been used for the 13 crops with over 100 beneficial traits having been derived from them (Table 3).

Table 3. Use of crop wild relatives in the past 20 years in released cultivars of 13 crops of international importance (Reem and Toby, 2017)

Crop	Pest and disease resistance	Abiotic stress	Yield	Quality	Male sterility or fertility restoration	Total number of contributed traits
Cassava	+	–	–	+	–	3
Wheat	++++++++ +++	–	+	+	–	9
Millet	+	–	–	–	+	3
Rice	+++++++	+++	+	–	+	12
Maize	+	–	–	–	+	2
Sunflower	+++	+	–	–	+	7
Lettuce	+++	–	–	–	–	2
Banana	++	–	–	–	–	2
Potato	++++++	–	–	–	–	12
Groundnut	+	–	–	–	–1	1
Tomato	++++++++ ++	++	–	++	–	55
Barley	–	+	–	–	–	1
Chickpea	–	+	–	–	–	2

+ : number of wild relatives that have contributed beneficial traits to crop varieties in each category of traits;
–: wild relatives have not contributed beneficial traits in that category.

Over 40 resistant genes are derived from *Lycopersicon peruvianum* L. Mill., L. *cheesmanii* Riley, *L. pennellii* (Correll) D'Arcy, and many other wild relatives of tomato for traits such as increased soluble solid content, fruit color, and adaptation to harvesting (Rick and Chetelat 1995). These improvements are reviewed in the Prescott-Allen and Prescott Allen (1986) study. Since then, QTL mapping and analysis has aided the discovery of useful quality-controlling genes, such as fruit size, in unlikely candidates such as the small-fruited tomato ancestor *L. pimpinellifolium* (Tanksley and McCouch 1997). In potatoes, resistance to late blight incorporated from wild species *Solanum demissum* and *S. stoloniferum* Schltdl. and Bche ´is still effective in some areas.

Wild relatives of cultivated crops are the raw material for plant breeder. Recently the importance of CWR has been realized globally to breed climate resilient crop varieties to meet out the future food security. CWR's has many fold applications in crop improvement which include different traits such as biotic stresses (pest /disease resistance, yield, quality, and male sterility) and abiotic stresses (heat, flood, cold and drought tolerance). To develop trait- specific genotypes, the CWRs have been utilized with varying degrees for significant traits. At present, most of the varieties (65%) released for commercial cultivation are either direct selection from germplasm or developed (20%) using trait specific germplasm as one of the parents in hybridization program.

At NBPGR, a total of 228 lentil interspecific derivatives of *Lens ervoides* and *L. orientalis* wild lentil species with cultivated *L. culinaris* are being advanced (through single seed descent method) to next generation. Short Internode and high seeds plant^{-1} from *ervoides* species and high number of pods plant^{-1} from *orientalis* species are the traits transferred to the cultivated lentil varieties. The cross combinations are ILL10829 × ILWL30 and ILL8006 × ILWL 62 respectively. These derivatives currently are in F_9 generation. These derivatives were also evaluated for variation in agro-morphological traits like leaf colour, leaf pubescence, leaflet size, flower ground colour, pod dehiscence, days to 50% flowering, days to 80% maturity, plant height and yield traits like branches/plant, pods/plant, biological yield/plant, 100 seed weight, number of seeds/plant, seed yield/plant and harvest index. Many recombinant inbred lines in each cross type have been identified promising for their respective traits.

Table 4: Pre-Breeding work in different crops

Crop	Attempt	Reference
Bean	Wild relatives are a potential source of novel alleles that can be exploited for the improvement of yield and other quantitative traits.	Acosta-gallegos *et al.*, 2007
Soyabean	Useful traits have been identified and introgressed in cultivated species from wild species through inter-specific hybridisation	Sebolt *et al.*, 2000)
Rice	Continuous efforts are being made to transfer the desired traits into the cultivated varieties from the rice accessions which are stored in gene banks due to narrow genetic base	Plunkett *et al.*, 1987
Maize	Value of exotic resource has yet to be explored in polymorphic genome which is resulted due to gene flow between cultivated and wild species	Cantrell *et al.*,1996 And Wang *et al* 1999, Luciano and Peterinain 2000, Nass and aterniani, 2000
Tomato	Different genes for disease resistance have been incorporated from various wild resources in commercial hybrids through recurrent back cross and each resides on a small independent chromosome segment from one of the diverse donor species. An important gene was introduced from the wild tomato species (*Lycopersicom ennellii* B.) resulted into raised level of Pro-vitamin A in the fruit by more than 15 fold	Ronen *et al.*, 2000
Pea	Improved the existing cultivars using wild species for resistance to biotic stress and abiotic Stress and quality improvement	ICRISAT, 2014

The interspecific crosses between PLM 96 x VRB 3 were developed successfully in mungbean (*Vigna radiata* (L.) Wilczek) as female parent and Rice bean (*Vigna umbellate* (Thunb.) Ohwi and Ohashi) to transfer MYMV resistance with the help of embryo rescue technology.

Challenges and future prospects of pre-breeding

The major challenges of pre-breeding for utilization of plant genetic resources are lack of characterization, evaluation of genetic diversity, documentation of data; inter species relationship and strong breeding program and funding sources. The above mentioned problems draw the attention towards urgent need for collection, characterization and documentation of wild species, including crop wild relatives, due to increased likelihood of extinction for narrowly adapted and endemic species. There is a rise in demand of novel genes in germplasm/ gene banks collections to make the agriculture tolerant/resistant against biotic and abiotic odds. Genome mapping, decoding of genes and synteny among the genes could be assigned to conceal the stress tolerance and can be utilized for Crop improvement. Therefore, the immediate need is to increase the use of plant genetic resources especially CWR through conventional, novel, and emerging approaches due to increased likelihood of extinction for narrowly adapted and endemic species. The blend of modern technologies can increase the chances of locating novel genes within natural occurring population of wild species, and their introduction in developing new climate smart crop varieties. Consequently, the collection, conservation and exploitation of plant wild genetic resources would definitely help increase the income, food and nutritional security of all.

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14

Advances in Plant Genetic Resources Evaluation

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"Plant genetic resources are the taxonomic and genetic diversity of plants of value as a resource for the present and future generations of people" (IPGRI, 1993). To enhance the plants of value of resources for utilization of germplasm the accessions conserved in the genebanks should be characterized to facilitate preliminary selection of germplasm by the researchers. The process of 'Characterization' describes plant germplasm by determining the expression of highly heritable morphological, physiological or agronomical characters or by using DNA markers. It facilitates easy and quick discrimination between accessions. Characterization of germplasm is normally the responsibility of genebank curators. The term 'Evaluation' refers to the description of germplasm for multi-genic characters that are important in breeding programme. It is usually done for traits such as yield performance, biotic and abiotic stresses and biochemical traits.

The expression of these traits is usually influenced by the environment and therefore may require special experimental designs and techniques. Evaluation is usually done by a multidisciplinary team of scientists which usually includes breeders and specialists e.g. entomologist for insect resistance, physiologist for stress tolerance, pathologist for disease resistance, etc. In practice, characterization and preliminary evaluation are usually done during the initial seed increase or the first regeneration cycle. Characterization and evaluation start with the adoption of a descriptor lists developed by the CGIAR and national institutions. A descriptor list is a collation of all individual descriptors used for a particular species. Many species have a standardized, agreed descriptor list. A descriptor is an identifiable and measurable trait or characteristic of a plant accession (e.g. height; color) used to make classification, storage, retrieval and use, more uniform. Each descriptor has variants of expression known as descriptor states. Several important species have a standardized descriptor list published by the former International Board for Plant Genetic Resources (IBPGR) and International Plant Genetic Resources Institute (IPGRI), now Bioversity International. Good characterization and evaluation data in a well organized documentation system leads to enhanced utilization of germplasm.

Lessons to learn

- ✓ Complete characterization and evaluation of accessions conserved in the genebanks ensure the effective utilization of the germplasm collection.

- ✓ Understanding of the taxonomy, agro-morphology and genetics of the crop species is essential for characterization and evaluation.
- ✓ Characterization and evaluation are indispensable to estimate the extent of diversity exists in genebank collections and determine gaps in the collection.

Methods of characterization and evaluation

There are various ways to describe the germplasm includes morphometric, biochemical and molecular approaches. Morphological characterization deals with description of an accession based on morphological markers taken at the various stages of growth (seedling, vegetative, inflorescence, fruit and seed). Stable and unique morphological traits should be effectively used for assessing the degree of genetic variation in the initial as well as regenerated *ex situ* conserved germplasm. Molecular characterization of germplasm accessions is a useful tool for better management and to study genetic diversity and integrity of conserved germplasm. Molecular markers are readily detectable sequence of DNA or proteins whose inheritance can be monitored. There are several methods that can be employed in molecular characterization, which differ from each other in terms of ease of analysis, reproducibility, level of polymorphism, number and genome distribution of loci. Previous studies using molecular tools have been performed on the genetic integrity of genebank accessions of some crop species during regeneration. Single Nucleotide Polymorphism (SNP) is widely used in different crops *viz*., barley, maize etc. Fingerprinting of genebank accessions aid in management of genetic integrity of the germplasm accessions as well as the molecular diversity (Mishra *et al*., 2006).

Importance of characterization and evaluation

- ✓ reveal information for an accession;
- ✓ maintain the original population of an accession over a number of regenerations;
- ✓ locate specific and unique traits;
- ✓ estimate genetic diversity of the collection;
- ✓ fingerprint genotypes;
- ✓ recognize duplicates;
- ✓ determine gaps in the collection;
- ✓ facilitate preliminary selection of germplasm by end-users;
- ✓ study genetic diversity and taxonomic relationships;
- ✓ develop core, mini-core collection and reference sets

Management of plant genetic resources

Core collection

Frankel (1984) termed 'core collection' which would represent 'with a minimum repetitiveness, the genetic diversity of a crop species and its wild relatives. Size of the core is approximately 10% of the whole collection (Figure 1). Large size of collections and lack of reliable data on traits of economic importance, which show high genotype × environment interaction are considered as the main reasons for low use of genetic resources. Core and mini-core collections based on passport, characterization and evaluation data and reference sets based on genotyping information are developed to enhance utilization. Due to its reduced size and representativeness of species diversity, the mini-core collections are emerged as ideal genetic resources for in depth characterization of its biological diversity and use in crop improvement programmes.

Advantages of a core collection

- Addition of new accessions: A core set provides a ready reference set of accessions for assessing redundancy.
- Conservation: The core contains materials which is highest in priority for conservation.
- Characterization and evaluation: The core set is appropriate for developing an adequate list of descriptors. Core set has maximum use in evaluation of germplasm.
- Germplasm enhancement: The breeding of desirable characters from alien genetic background into locally adapted materials is a lengthy and expensive process.
- Germplasm distribution: Development of a core set can help to accelerate the response to requests of various indentors.

How to select core entries and develop a core collection?

Steps:

1. Classification on the basis of taxonomic status ((species and cytotype)
2. Further classification on the basis Category of material (wild, landrace, odd or recent, cultivar, breeder line)
3. Grouping as per Region, Country, etc.
4. Morphological traits
5. Validation of core

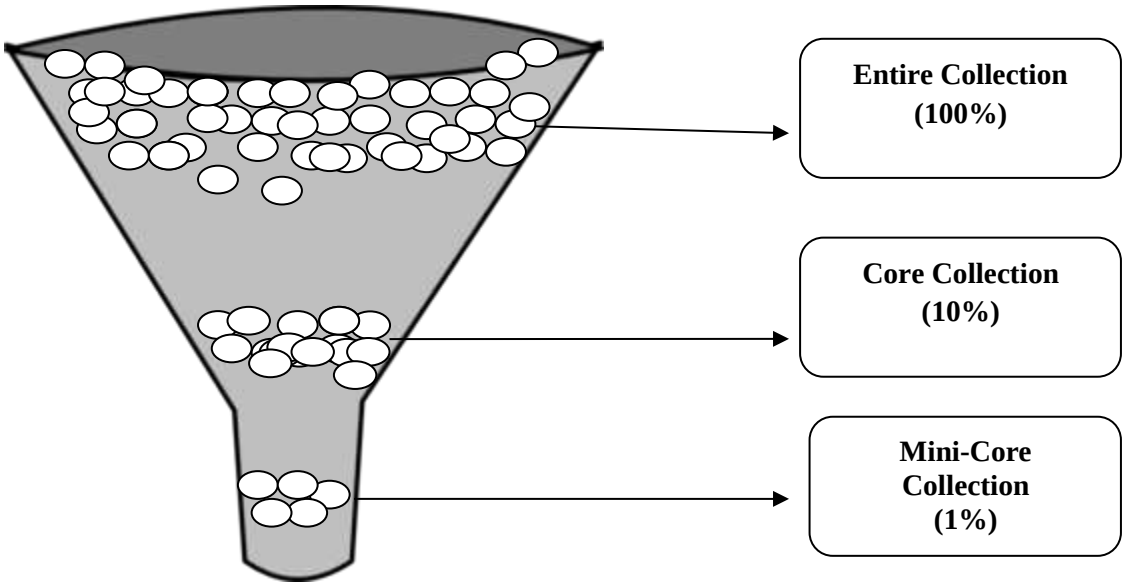

Figure 1: Core and mini-core collection

Pre-breeding

Characterization and evaluation of wild species is necessary to utilize these alien genetic resources for strengthening pre-breeding activities. Pre-breeding is the identification of desirable traits from unadapted materials that cannot be used directly in breeding populations, and to transfer these traits to an intermediate set of materials that breeders can use further in producing new varieties for farmers. The pre-breeding increased access and use of genetic variations conserved in genebanks which directly facilitates the efficiency and effectiveness of crop improvement programmes.

***Applications of pre-breeding in crop improvement*:**

Pre breeding mainly applied in four major aspects:

1) Narrow genetic base results into the crop vulnerability to different biotic and abiotic stress. Pre breeding is adopted for broadening the genetic base, to reduce vulnerability.

2) Identifying desirable traits in exotic materials and moving those genes into material more readily accessed by breeders.

3) Wild species and crop wild relatives are the reservoir of the gene for cope with the changing climate, identification of this important gene and moving them from wild species into breeding populations when this appears to be the most effective strategy.

4) Identification of novel genes in the unrelated species and transfer them using genetic transformation techniques.

Accelerating genetic gain through use of genetic resources

Researchers are now using high throughput phenotyping and genotyping platforms (including genotyping by sequencing) to dissect the genetic and physiological basis of trait expression and deployment to accelerate genetic gains in crop breeding. In addition, researchers are now sequencing genomes of diversity panels and its comparison with reference genome is expected to associate sequence difference with agronomically beneficial traits. The judicious use of these genetic and genomic resources have enabled researchers identify significant marker-trait associations or candidate genes associated with agronomic traits, for example, stress tolerance and agronomic traits in sorghum, chickpea and groundnut. In addition, the use of groundnut germplasm lines identified from mini-core collection have resulted in developing exceptionally high oil (up to 63%, compared to ~48% in control cultivar) and high-yielding breeding lines, indicating that new germplasm sources contribute to enhancing genetic gains. In conclusion, plant genetic resources play an important role in developing cultivars with high genetic gains to meet the adverse effects of climate change on global agriculture. Use of representative subsets such as mini-core collections serves as an ideal diversity panels for trait discovery. The availability of high throughput genotyping and phenotyping facility has opened the gateway to accelerate understanding of the genetic control of the trait expression and subsequently to rapid genetic gains in crop breeding (Upadhyaya, 2016).

Enhanced utilization of germplasm in crop breeding programmes

An analysis of the uptake of germplasm in crop improvement programmes at ICRISAT showed that germplasm use has increased since the formation of the mini-core collections in some crops. For example, 30% stresses tolerant accessions used in breeding during 2005-2009 were from mini-core, while in 2010-2014, more emphasis (17%) was given to those germplasm possessing yield and seed nutritional traits. The emphasis in groundnut during 2005-2009 was on stress tolerance (37% from mini-core), which during 2010-2014 changed to involving both stress tolerance (54%) and yield and quality (52%) traits in breeding.

Phenotyping of germplasm

Now, advanced phenotyping is being done by using phenomics facility. The word 'phenome' refers to the phenotype as a whole i.e., expression of genome for a trait in a given environment while in phenomics, we get high-dimensional phenotypic data on an organism at broad scale. Actually phenomics is parallel but differs from genomics. In genomics, complete characterization of a genome is possible while in phenomics, complete characterization is difficult due to the change in the phenotypic expression of traits over the environmental conditions (Kumar *et al*., 2015).

Phenotype vs Phenomics

Phenotype of a plant can be described on the basis of morphological, biochemical, physiological and molecular characteristics. After 1950, 'phenotyping' as a noun, 'to

phenotype' as a verb and 'phenome' as the collective noun were introduced, which have been accepted scientifically and are being utilized commonly in literature. Using phenotyping tools, forward phenomics discriminate the useful germplasm having desirable traits among genebank collections. This results to identification of the 'best of the best' germplasm. Use of high-throughput, fully automated and low resolution followed by higher-resolution screening methods have accelerated plant breeding cycle by screening a large number of plants at seedling stage. Thus interesting traits can be identified rapidly at initial stage and there is no need to grow plants up to the maturity stage in field (Figure 2). Now it is indeed possible in forward phenomics to screen thousands of plants in pots running along a conveyor belt, and travelling through a room containing automated imaging systems such as infra-red or 3D cameras. The pots are labelled with barcodes or radio tags, so that the system can identify which pots contain plants with interesting traits. The selected plants can then be grown up to produce seed for further analysis and breeding. The reverse phenomics is used where the best of the best genotypes having desirable trait(s) is already known. Now through reverse phenomics, traits shown to be of value to reveal mechanistic understanding are dissected in details and subsequently the identified mechanisms are exploited in new approaches. Thus in reverse phenomics, we discover mechanisms which make 'best' germplasm the best. This can involve reduction of a physiological trait to biochemical or biophysical processes and ultimately a gene or genes. For example, in case of drought tolerance, researchers try to work out the mechanisms underlying the drought tolerance and find out the gene or genes that are responsible for it. These genes are screened in germplasm or the gene can be bred into new varieties.

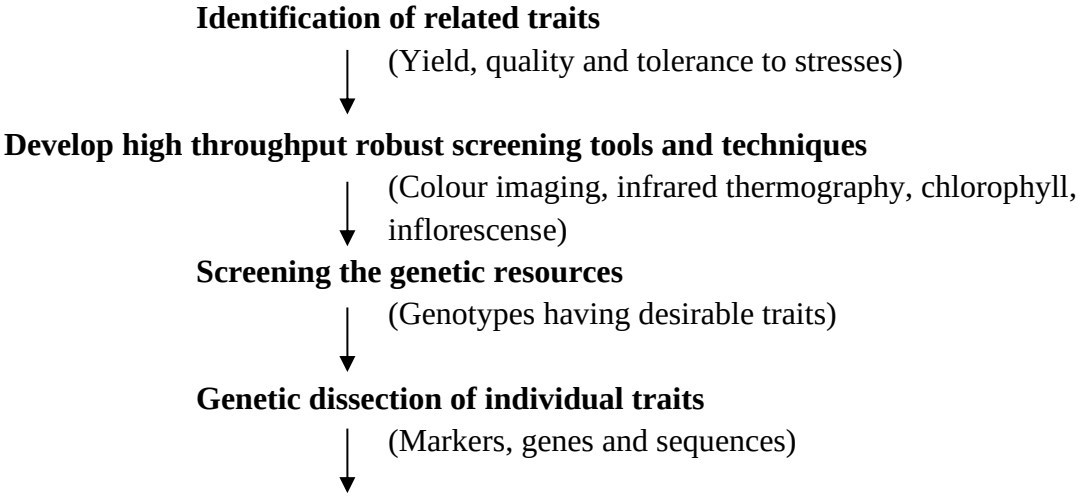

Figure 2: Flow chart of application of non destructive phenotyping in genetic dissection of traits

Evaluation for biotic stresses

The screening for resistance to pests is a complex process as it involves the three complex organic systems interacting in various ways. These are host plant, pest and the environment. The success of the identification of resistant/ tolerant germplasm will largely depend upon the methodology adopted to screen such trait specific germplasm (Tripathi *et al.*, 2018).

Basic considerations for screening

- Plant material with diverse genetic base provides better chances of locating desirable sources of resistance. If varied germplasm, including from the place of origin of the crop is available, the chances of locating stable sources of resistance are more.
- The crop curator is dealing with a range of biotic stresses and it is not practically feasible to screen the germplasm against all the pests. Hence, number of stresses to be evaluated, must be prioritized based on its impact on growth parameters, yield of a crop and economic significance.
- Evaluation of germplasm against biotic stress means evaluation against the pathogen or pest not against the disease because the similar disease symptom may be produced by different pathogens. So, it is essential to identify the pest. As per the identified pathogen or pests, mass culture of such pathogen or pests needs to be done. In case of viruses, infectious viral construct needs to be prepared for inoculation purpose.
- Inclusion of control plants of known susceptible phenotype for screening programme provides assurance that many variables of the environment, pest pathogen and the host are operating with the range that will provide a satisfactory interaction phenotype.
- Identification of pest is also important to know the race/strain/ isolate/biotype which exist at that particular location. Identification of resistance source against a particular race/strain/isolate/ biotype within a particular location does not guarantee its resistance response in other locations as race/strain/isolate/ biotype may vary depending upon the agro-meteorological conditions. So screening of germplasm for biotic stresses should be accompanied with identification of race/ strain/isolate/biotype of the pest.

In screening plant germplasm of unknown resistance, care must be taken to ensure that the exposure to disease potential occurs at the appropriate stages during plant development. The initial evaluation of germplasm is done in the field under epidemic conditions with as broad a representation of pathogenic variation and favourable environmental conditions for epidemic development as possible. The most common method of disease assessment is visual estimation of disease tissues. Assessment may be of many characteristics such as incidence lesion type, percentage leaf area affected, or percentage of the entire plant that shows symptoms. The simplest evaluation can be made when resistance is clearly expressed. The most apparent method is the use of a rating scale. Scales usually run from 1-4, 1-5 or 1-10 but other variations are known such as the 0-9 scale used for cereal mildews. The chosen scale should be clearly defined to indicate resistant or susceptible entries. For general germplasm evaluation, there are published scales for large-scale germplasm evaluation. Evaluation of germplasm against soil

borne pathogens: Characterization procedures for evaluation of resistance of plant germplasm to root diseases are generally difficult. Symptoms of root decay are the results of a continuous interaction between the microbe and the host. Subjective severity-rating scales are commonly used to describe extent of necrosis or root decay, e.g., a 0-5 scale of root discoloration (decay) with 0= healthy root and 5= maximum severity including complete necrosis of all roots. Intermediate ratings may occur whenever decay is inhibited (metabolic resistance) or when the roots per plant 'escape" infection (containment of infection, regeneration of root system etc.). It should be done for one year with large number of accessions to narrow down the numbers to a manageable extent. For such purpose only percent disease incidence should be recorded and field tolerant accessions (<20% incidence) are selected.

Advanced field screening of the selected field tolerant accessions should be done at least for two years. The advanced screening plot and germplasm maintenance plot should be different. In advance screening plot no plant protection measures are adopted so that the germplasm is exposed to the maximum load of inoculum pressure of pathogen or vectors of viruses. All the experiments are done in replicated trials with susceptible check that can serve as infector row, preferably one line of infector row after every three lines of germplasm. The data should be recorded at regular intervals.There should be border rows of susceptible check in all the directions to facilitate spread of the disease infestation. For recording of the data on fungal and bacterial foliar diseases, standard evaluation system (SES) scale should be followed. For recording of the data on viral diseases, incidence (number of plants showing symptom over total number of plant) and average disease severity (number of leaves showing symptom or area covered over total number of leaves in a single plant and averaged from at least five plants) should be taken into account. A severity index grade is formulated for individual disease based on the progress of the disease and coefficient of infection (CI) is calculated using the formula: CI=% disease incidence (PDI) X response value (severity grade). As per the CI value, a scale is prepared and the response of the germplasm is categorized. The area under disease progress curve (AUDPC) value is calculated to understand the rate of spread of the disease. The germplasm with low CI and AUDPC value should be selected as promising accession. For soil borne pathogen, the selected germplasm should be grown in sick plot with optimum inoculum load. The susceptible check should show at least 80-100% infection to confirm the optimum inoculum load. • For air-borne pathogen, there should be spraying of spore suspension of the pathogen at regular intervals. In this case also, the susceptible check should show at least 80-100% infection to confirm the optimum inoculum load.

Greenhouse Screening: Following the identification of effective resistance in the field, the screening methods are devised that would evaluate the potential for that resistance to be expressed under the more controlled environments. In addition to enabling greater precision in the production of IP through the control of environment and the delivery of inoculum, laboratory methods may provide the opportunity of screening for multiple disease screening. The success of laboratory methods will depend on whether the particular resistance expressed in the field, is also expressed at other stages in the plant development. Expression of resistance

is sometimes highly tissue specific and restricted to certain organs or to expression at certain developmental or physiological stages of plant growth. For virus and other vector borne pathogens, advance screening under natural field condition are carried out for two years and the promising accessions are challenged under controlled glasshouse conditions with viruliferous insects (for insect transmitted virus), sap from the infected plant (for mechanically transmitted virus) and grafting (for the viruses transmitted by vegetative propagules). Artificial inoculation under controlled conditions should also be carried out for fungal and bacterial pathogens. For such experiments advanced facilities like temperature humidity controlled glasshouse, insect maintenance chamber, plant inoculation chamber etc. are essentially required. Finally, the identified accessions may be tested under multi-location evaluation at hot-spots to evaluate its performance against other race/strain/isolate of the pathogens that may exist in different locations.

FIGS

The focused identification of germplasm strategy (FIGS) is an efficient approach to explore agro-biodiversity for climate change adaptive traits. one such approach. FIGS works on the premise that germplasm is likely to reflect the selection pressures of the environment in which it developed. Environmental parameters describing plant germplasm collection sites are used as selection criteria to improve the probability of uncovering useful variation. FIGS has powerful algorithm that matches plant traits with geographic and agro-climatic information of the places where samples were collected (Khazaei *et al*., 2013). This allows the rapid searching of thousands of plant samples conserved in gene banks to pinpoint a number of high potential types that can meet the breeder's strategy. It creates 'best-bet' trait-specific subsets of materials by passing accession-level information, especially agro-climatic site information, through a series of filters that increase the chances of finding the adaptive trait of interest. Accessions from these areas have a higher probability of containing traits and genes of interest. From this calculation, smaller subsets of genetic material are assembled that have a high potential of containing the plant traits that breeders need to develop robust crop varieties – capable of tolerating drought and high temperatures and resisting pests and disease. In several hundred searches delivered to date, FIGS has demonstrated that it can identify specific traits for breeders rapidly and precisely. In some cases it has identified traits that researchers have been looking for, unsuccessfully, for a number of years. Examples given below:

- FIGS achieved a 16 percent 'success rate' in identifying genotypes resistant to powdery mildew disease, compared to the 5 to 6 percent typically obtained with traditional screening methods.
- FIGS identified the first-ever sources of resistance to the most virulent biotype of the Russian wheat aphid.
- FIGS analysis revealed new resistance genes to mildew and aphids, that are expected to significantly improve resistance breeding programs.

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Role of Genomics in PGR Management

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Plants are the basic support system of life and therefore conservation of their genetic resources for sustainable use to feed tomorrow's population is of utmost importance. The genome is an organism's complete set of DNA. The omics era has brought forth 'Genomics' as an interdisciplinary field of biology relating to the structure, function, evolution, mapping, and editing of genomes. Genomics has a considerable role to play when it comes to management of the Plant Genetic Resources (PGR). The PGR describe variability within plants whether it comes from natural selection or through human intervention, over the time. Taking the way forward from the application of first generation DNA based markers such as Restriction Fragment Length Polymorphism (RFLP), to PCR based markers such as SSRs, to the recent breakthroughs in the sequencing based technologies, genomics has played a major role in the management of the plant genetic resources and their utilization (Figure 1).

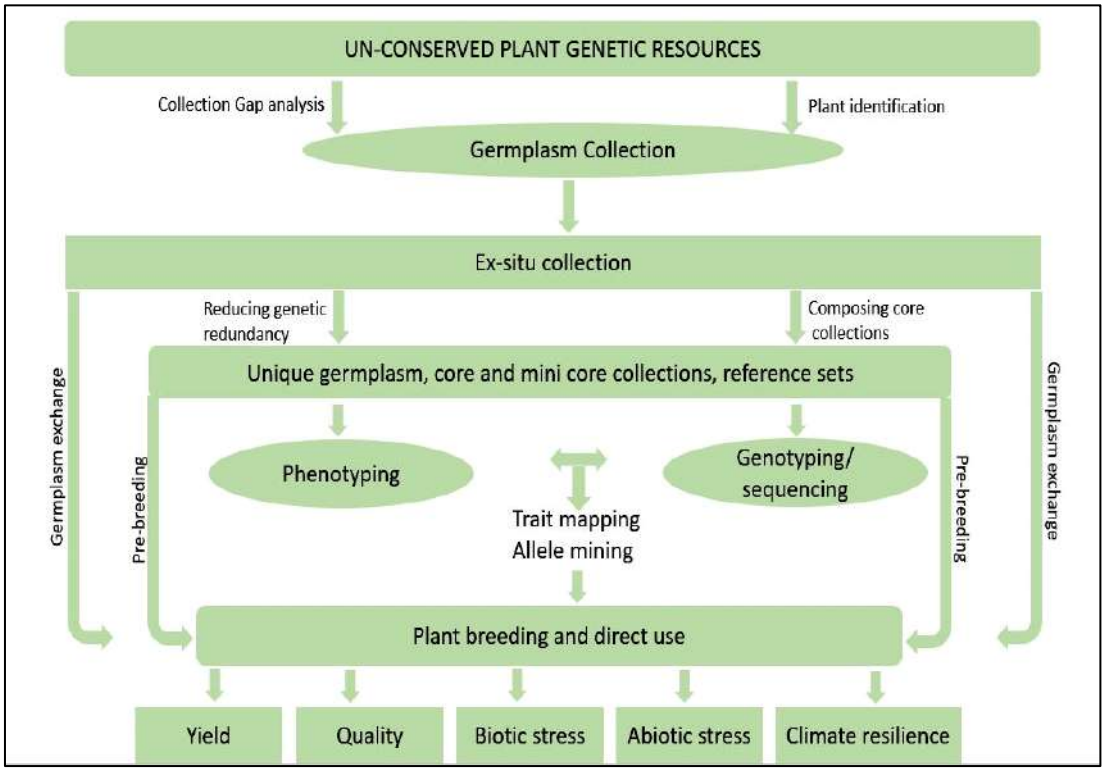

Figure 1: Schematic representation of potential application of genomics in germplasm utilization (Wambugu *et al.*, 2018)

Next generation sequencing technologies have revolutionized research by allowing an economic elucidation of the genome of any organism. Genome projects of any selected plant species encompass three major elements: DNA sequencing of whole genome, assembly of the reads representing genome, annotation and analysis of the assembled data. The technology has found application in many areas, and PGR management is not an exception. Crop germplasm resources are the raw material for crop improvement programs leading to development of varieties that fulfil the food requirement of the world. Increased population, reduction in arable lands and environmental degradation combined with biotic and abiotic stresses and erosion of genetic pools have made sustainable exploitation of plant genetic resources a key to the new green revolution that aims towards higher productivity, lower inputs and improvement of human nutrition.

Assessment of genetic diversity is a prerequisite for analyzing as well as utilizing the variability occurring in a cultivated species and its wild and weedy relatives. At NBPGR, generation, validation and utilization of genomic resources is the major objective of the Division of Genomic Resources. These resources are utilized for value addition to the plant germplasm resources harbored in the gene bank and for generating molecular profiles varieties of agri-horticultural crops. The availability of NGS technologies has made possible analyzing a large number of genebank accessions thereby targeting maximum allelic variability. The combination of genomics and conventional genetic approaches has led to the development of "genomics- based germplasm research" (GPGR) that encompasses the characterization and utilization of plant germplasm using these tools. The concept of development of a core collection that defines a minimum set of accessions capturing the maximum proportion of genetic diversity is primary to any genebank curator. Subsequent to the identification of a core collection based on morphological and geographical information, the use of molecular markers in further reducing the core to a minicore has been adopted as a strategy for many crops. Further characterization of the minicore to an applied core collection using the focused identification of germplasm strategy (FIGS) has been accelerated using the next generation sequencing technologies. These include characterization of the germplasm using low coverage sequencing strategies such as genotyping by sequencing (GBS) with the advantage that a large number of samples can be multiplexed at low cost inputs. The identification of high throughput Single nucleotide polymorphisms (SNPs) the ultimate molecular markers, through NGS, has facilitated the characterization of germplasm, particularly for crops like rice and wheat.

Gene discovery, the final goal of any breeding programme has been greatly accelerated through the genomics tools using map based, genome wide association studies (GWAS), allele-mining and comparative genomics approaches. The utilization of NGS technologies on the characterization of germplasm has opened up a plethora of information for the efficient and sustainable utilization of these invaluable resources.

Genomics thus has a wide application on the conservation and utilization of genetic resources and will restructure the way of managing gene banks. In addition to understanding the diversity

of plant species, genomics approaches enable studying gene duplication and identifying lateral gene transfer, one of the driving forces of evolution. The current genotyping and sequencing techniques and the value addition to the genebanks through the genomics approaches have thus minimized the challenges that limit germplasm utility.

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DNA Fingerprinting of Crop Varieties

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Genomic DNA constitutes the total genetic information of an organism. The genomes of almost all organisms are DNA, the only exceptions being some viruses that have RNA genomes. Genomic DNA molecules are generally large, and in most organisms are organized into DNA–protein complexes called chromosomes. The size, number of chromosomes, and nature of genomic DNA varies between different organisms. Viral DNA genomes are relatively small and can be single- or double-stranded, linear, or circular. All other organisms have double-stranded DNA genomes. Bacteria have a single, circular chromosome. In eukaryotes, most genomic DNA is located within the nucleus (nuclear DNA) as multiple linear chromosomes of different sizes. Eukaryotic cells additionally contain genomic DNA in the mitochondria and, in plants and lower eukaryotes, the chloroplasts. This DNA is usually a circular molecule and is present as multiple copies within these organelles.

DNA fingerprinting

DNA fingerprinting involves the generation of a set of distinct DNA fragments from a single DNA sample. The generated DNA fragments are then used as a source of genotypic information. A variety of techniques can be used to generate DNA fingerprinting patterns. The choice of the technique depends on the organism being studied and on the question being addressed. All DNA fingerprinting techniques study patterns associated with genetic markers; however, individual techniques differ in terms of the number and type of genetic markers examined. For example, some approaches allow the examination of a marker at a single locus (called single-locus markers), whereas others allow the simultaneous investigation of multiple loci (called multi-locus markers). Some approaches focus on co-dominant markers, which provide information about both alleles present at a given locus. In contrast, other techniques are concerned with dominant markers, which only report the presence or absence of a given allele and cannot provide information about whether an individual is homozygous for that allele.

In 1985 the concept of a "DNA fingerprinting" was introduced as a means of evaluating human identity and relatedness (Jeffreys and Wilson, 1985). The possible forensic and legal applications of DNA evidence were quickly appreciated and such data are now frequently presented in court cases involving serious crimes such as murder and rape. DNA evidence is also used in establishing paternity, in determining relatedness in immigration and inheritance disputes, and in identifying disaster victims. Such cases, especially those involving famous

people, are widely reported in the media and are of interest to the general population. DNA fingerprinting technology has now been extended from humans to even plants. In plants, identification of cultivars is one area where DNA fingerprinting is being used routinely and has applications in protection of plant breeders' and farmers rights.

Cultivar fingerprinting

The question underlying the use of DNA fingerprinting data is what is the probability that two or more cultivars have the same genetic fingerprint/ profile?

If we could sequence and compare the DNA of each cultivar, we would find that all cultivars are different. Even natural clones, such as identical twins in humans, will have acquired a few differences that are mutations in their DNA, during their growth from a single cell to an adult. When it is not possible to sequence the entire individual's genome then instead, we rely on differences in length of short stretches of DNA at different loci commonly called markers. These stretches of DNA can be visualized as shown in the figure. Based on this figure can we say that any two cultivars are identical? Can we assign a numerical value to our answer? Our answer to this question depends on the probability of two or more cultivars having the same pattern of these DNA fragments, the same sized SSR markers. The SSRs chosen for DNA fingerprinting purposes are inherited independently of each other. They are on different chromosomes. Therefore, in theory, if the frequency of SSR1 in a population is 1 in 50, and SSR2 has a frequency of 1 in 1000, then only 1 in 50,000 cultivars would be expected to have both these SSRs. More SSRs (not two as in the present case) are usually monitored in each "DNA fingerprint" and are chosen so that the probability of a false positive match is extremely low (Figure 1).

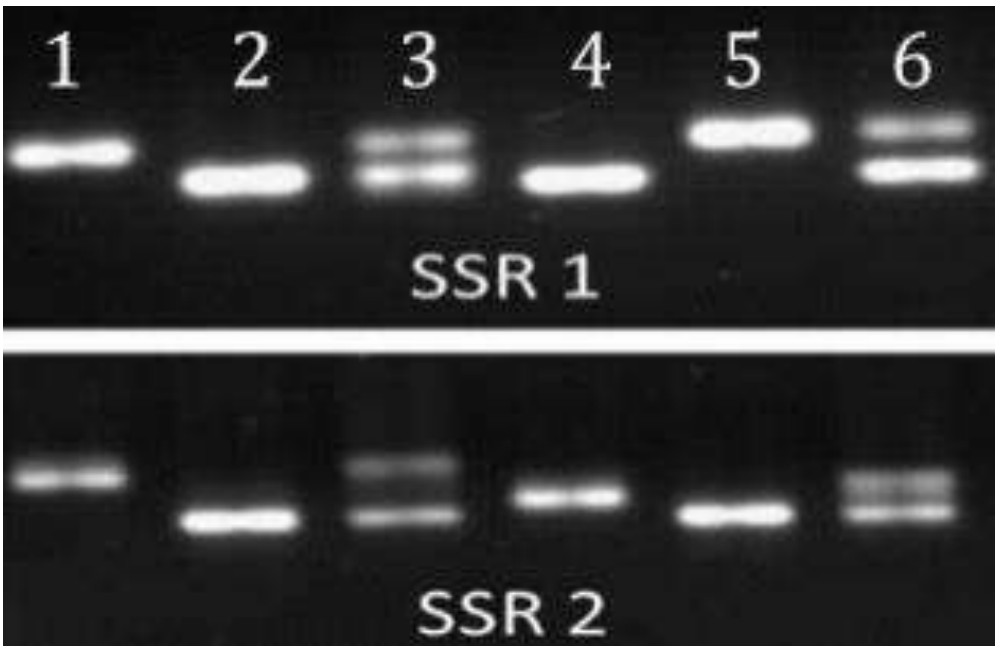

Figure 1: DNA profile, with SSR markers, of six cultivars with two primers

Choosing an appropriate methodology

There are different methodologies available for DNA fingerprinting such as RFLP, RAPD, ISSR, AFLP, SSR, SNP etc. The important criteria for choosing a methodology depends on the reproducibility of the data between laboratories, accessibility of detection platforms, repeatability over time, discrimination power and ease in creating database.

Database generation

As advancements in methodologies and new equipment are routinely happening, the continued sustainability of database should be an important consideration. This is particularly true for DNA sequencing data. Also, earlier radioactively labeled primers and sequencing gels were used to produce DNA fingerprinting data which now can be done using fluorescent labeling followed by separation on high throughput detection systems. Repeatability and reproducibility are important in the construction, operation and longevity of databases and is very important in generating a centrally maintained database, populated with verified data from different sources.

Mapped markers

With the availability of robust techniques, the trend has been to use mapped SSRs (microsatellites) and may be sequencing (Single Nucleotide Polymorphisms, SNPs) in near future. Other genic or functional marker techniques which rely on DNA sequence information may also fulfill the above criteria but their use in DNA profiling of plant varieties needs to be explored. A marker or set of markers selected for DNA profiling should be polymorphic and have repeatability within and reproducibility between, laboratories in terms of scoring data. To help avoid markers that may be linked, it is desirable to know the map position of the markers. It is also advisable to avoid markers with "null" alleles.

Microsatellite markers

Microsatellite or Simple Sequence Repeats (SSRs) make use of polymerase chain reaction (PCR) and has several advantages. SSR markers are expressed co-dominantly, are generally robust, repeatable, easy to score (record), and easy to automate for high-throughput detection. Moreover, several SSRs can be multiplexed and detected by non-radioactive DNA sequences or on gel electrophoresis or capillary electrophoresis. With recent advancements in genomics, mapped SSRs are now available in several crops. For effective microsatellite analysis, selecting high quality SSR markers that are unlinked, polymorphic, reliable and reproducible and having no stuttering is essential. Primers for any kind of marker system should be synthesized by an assured supplier. Some SSRs are publicly available.

Plant material

The source and type of the material and how many samples need to be analyzed are the main issues with regard to the material to be analyzed. The plant material to be analyzed should be

an authentic, representative sample of the variety and, where possible, should be obtained from the sample of the variety used for examination for the purposes of Plant Breeders' Rights or for official registration. Use of samples of material submitted for examination for the purposes of Plant Breeders' Rights or for official registration will require the permission of the relevant authority, breeder and/or maintainer, as appropriate. The plant material from which the samples are taken should be traceable in case some of the samples subsequently prove not to be representative of the variety.

Sampling strategies

The type of plant material to be sampled and the procedure for sampling the material for DNA extraction will, to a large extent, depend on the crop or plant species concerned. For example, in seed-propagated varieties, seed or seedlings may be used as the source of DNA, whereas, in vegetative propagated varieties, the DNA may be extracted from leaf material. Whatever the source of material, the method for sampling and DNA extraction should be standardized and documented. Furthermore, it should be verified that the sampling and extraction methods produce consistent results by DNA analysis. It is essential that the samples taken for analysis are representative of the variety. With regard to being representative of the variety, consideration should be given to the mode of propagation i.e. whether self-pollinated or cross-pollinated. The size of the sample should be determined taking into account suitable statistical procedures.

Reference set

A DNA reference sample set of varieties can be created for use in different detection equipment in different laboratories and for appropriate size calling as molecular size standards behave differently in the various detection systems. This DNA reference sample set can be stored and also supplied to other laboratories.

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Plant Genetic Resources Informatics

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What is PGR Informatics?

Plant Genetic Resources (PGR) Informatics is the management (creation, storage, retrieval and presentation) and analyses (discovery, exploration and extraction) of diverse information (facts, figures, statistics, knowledge and news).

Relevance of PGR informatics

Global assessment indicates that many of the world's PGR are insufficiently and poorly documented. The passport information and characterization and evaluation data on genebank accessions conserved in genebanks are either lacking or poorly recorded or scattered at different places, such as passport data sheets, reports of collection and exploration missions, crop catalogues, published articles, etc. In addition, there exist informal or non-coded knowledge held by traditional farmers and indigenous people. To use this information efficiently and effectively, the valuable information need to be collected, collated, maintained and exchanged with the help of PGR Informatics.The need for countries to develop, maintain and exchange information "from all publicly available sources, relevant to conservation and sustainable use of biological diversity" including "results of technical, scientific and socioeconomic research" has been recognized in the Convention on Biological Diversity (CBD, Articles 7d, 17), and the Global Plan of Action (GPA, priority activities 17 and 18). Information of this nature is imperative for planning and implementing activities; sustainable use and sharing of benefits accrued from its use.

Significance of PGR Informatics

Fundamental merit of an organized digital information system is that it provides fair and just opportunity for all to access. On-line portals, made available as a consequence of PGR Informatics, enable non-exclusive access to PGR information to a large number of users involved in overlapping research areas on PGR management. For list of global initiatives on PGR informatics databases and portals refer to Table 1.

Table 1: Global initiatives on PGR informatics: PGR Informatics databases and portals

Information resource	Web address
Barcode of Life	www.barcodeoflife.org
Convention on Biological Diversity	www.cbd.int
Encyclopedia of Life	www.eol.org
European genebanks portal	eurisco.ipk-gatersleben.de
Genesys portal	www.genesys-pgr.org
Global Biodiversity Information Forum	www.gbif.org
Indian Bio-resources Information Network	www.ibin.gov.in
Indian genebank portal	pgrportal.nbpgr.ernet.in
International Legume Database	www.ildis.org
International variety protection database	www.upov.int
Japanese genebank portal	www.gene.affrc.go.jp/databases_en.php
National Plant Germplasm System of USDA	www.ars-grin.gov/npgs
Species 2000	www.sp2000.org
World Information and Early Warning System	www.fao.org/wiews/en

Ever-increasing importance of PGR conservation and utilization on one hand and advancements in computer technology for digitization and management of data on the other have catapulted PGR Informatics into limelight. PGR Informatics has assumed significance because of the following factors:

- Increased awareness about PGRFA
- Various international agreements (CBD, GPA, ITPGRFA) coming into force
- Availability of information in text, images, maps, videos, etc.
- Technologies to record, link and archive such diverse types of information
- Growing power (and falling costs) of computers and internet to facilitate access and retrieval

Why manage PGR information?

The National Bureau of Plant Genetic Resources (ICAR-NBPGR) Plans, conducts, promotes, coordinates and provides the lead in activities of collection, conservation, evaluation, introduction, exchange, documentation and sustainable management of diverse germplasm of crop plants, crop wild relatives and landraces. Ever-increasing challenges to crop production

demands development of varieties that yield better in the face of biotic and abiotic stress. Crop improvement depends upon the availability of diverse germplasm.

PGR Informatics assists decision making on the prioritization and allocation of resources. Genebank curators, breeders and students as well as policy makers and funding agencies can rely on PGR Informatics tools and applications to plan and implement activities related to PGR management and utilization.

Typically, PGR Informatics manages accession level data including taxonomy, biogeography, and ethnobotany of the germplasm acquisitions (domestic collections and exotic introductions), their seed health, multiplication for supply and conservation, regeneration, experimental data on characterization and evaluation leading to utilization. In addition to field data, it also includes biochemical and genomic data as well as publications. Once the information is digitized and stored, computer technologies allow management and analysis irrespective of the scale and types of data leading better visualization and predictions.

Historical perspective

Biodiversity informatics as a discipline started with the construction of the first taxonomic coding system by researchers at the Virginia Institute of Marine Science for the Biota of Chesapeake Bay in 1972. This work led to development of a number of other taxonomic databases specializing in particular groups of organisms culminating into the "Catalogue of Life" in 2001 as well as into "Biodiversity Information Projects of the World." Encyclopedia of Life, Consortium for the Barcode of Life (CBOL), TreeBASE, Species 2000, Global Biodiversity Information Forum (GBIF), Inter-American Biodiversity Information Network (IABIN), World Biodiversity Information Network (REMIB), Indian Bio-resources Information Network (IBIN) inter alia have been the torchbearers of biodiversity informatics (Agrawal *et al*., 2012).

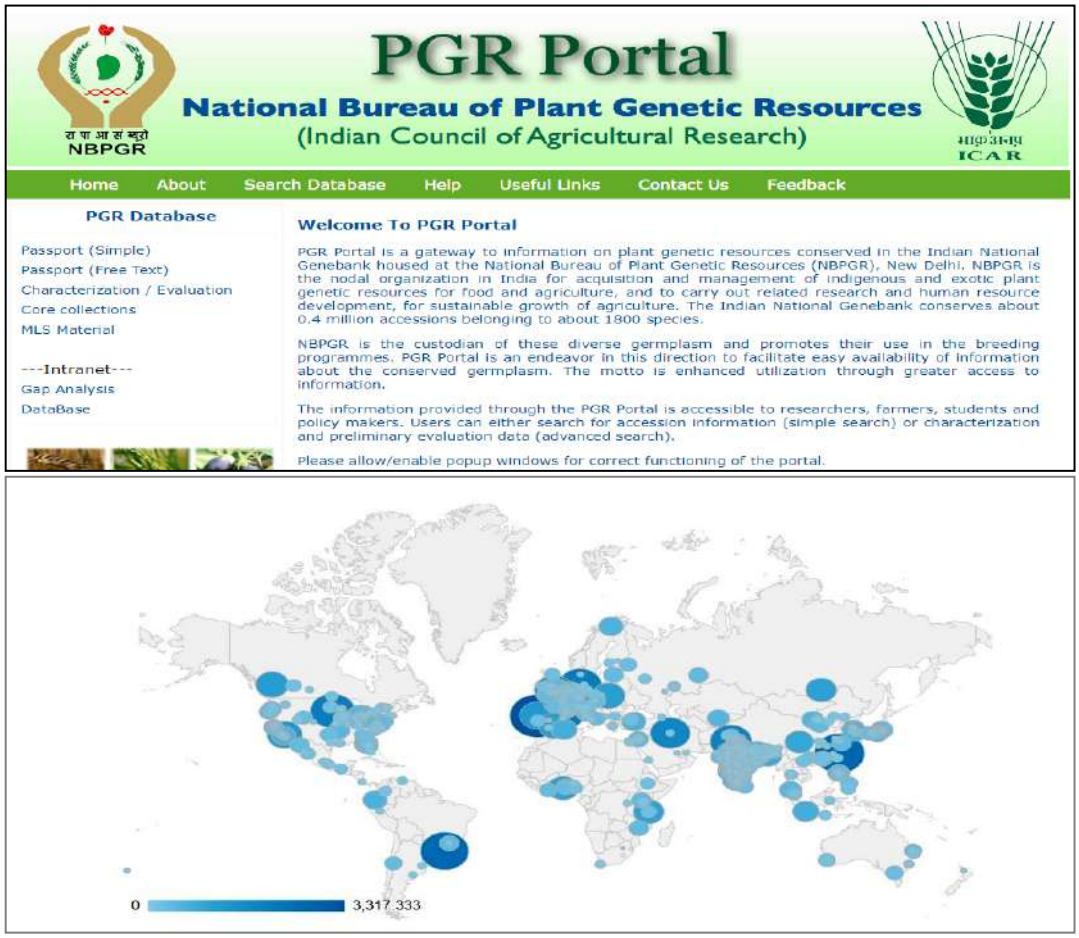
Germplasm Resources Information Network (GRIN) supports the national germplasm collections important to food and agriculture, collectively called the National Genetic Resources Program of United States Department of Agriculture. GRIN provides genebank personnel and germplasm users with access to databases that maintain passport, characterization, evaluation, inventory, and distribution data important for the effective management and utilization of national germplasm collections. *European Search Catalogue for Plant Genetic Resources (EURISCO)* is a search catalogue providing information about ex situ plant collections maintained mainly in Europe. It is based on a network of National Inventories of 43 member countries and 400 institutes providing information on ~2 million accessions. *NARO*is the Japanese Genebank of National Agriculture and Food Research Organization, manages databases that include information on passport data, evaluation as well as more general information on genetic resources. *GENESYS* is a global portal to information about PGR, from which information on germplasm accessions from genebanks

around the world can be found. GENESYS resulted from collaboration between Bioversity International on behalf of System-wide Genetic Resources Programme of the CGIAR, the Global Crop Diversity Trust and the International Treaty on the Plant Genetic Resources for Food and Agriculture. In addition to passport data, GENESYS provides access to over 11 million records of characterization and evaluation data. *PGR Portal*is a gateway to information on PGR conserved in the Indian National Genebank housed at ICAR-NBPGR, New Delhi, with information on about 400,000 accessions.

Indian initiatives in the field of PGR Informatics are concentrated at NBPGR. Following section lists major PGR Informatics applications that are developed at NBPGR and are being used by researchers around the world.

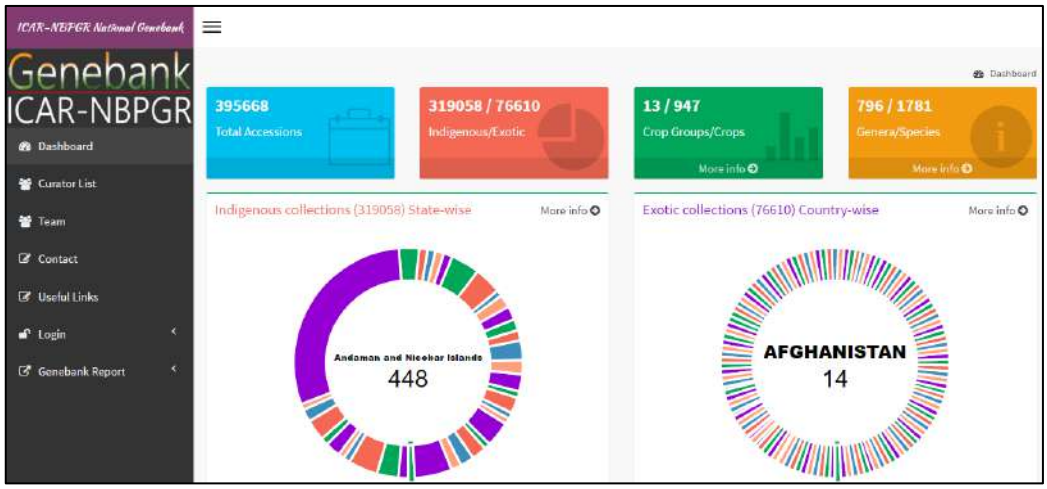
PGR Portal

PGR Portal is an open access information portal on plant genetic resources conserved in the National Genebank of NBPGR. Information on as many as 3,11,575 indigenous and 52,972 exotic accessions are accessible in the PGR Portal now. In addition to the continuous activity of testing and bug-fixing, addition of new features like "Gap analysis" and "Database" were added for internal activities.



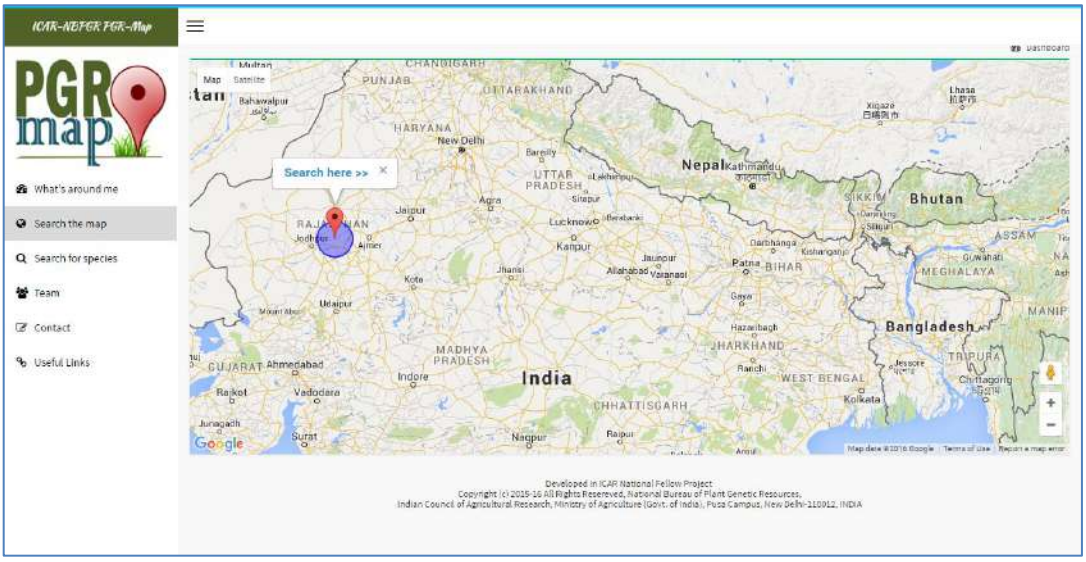
Genebank Dashboard

It is a user friendly Dashboard designed, developed and implemented to provide a personalized quick access to genebank information to PGR workers, breeders, students, research managers and administrators. The dashboard is designed to be compatible with old and new computers, tablets and smart phones. The dashboard figures are dynamically updated as and when genebank database is updated.



PGR Map

PGR Map offers map-based data retrieval providing easy and intuitive access to PGR information. PGR Map offers three benefits: *What's around me*, *Search the map* and *Search for species*.



Germplasm Exchange and Quarantine Information System (GEQIS)

Updated version of GEQIS was developed incorporating generation of new reports, enhanced data integrity by introducing automatic detection of data duplication, fee calculations and

invoice/receipt printing incorporating new GST rules, allowing indigenous (IC) entry along with exotic (EC) entries etc.

भाकृअप – राष्ट्रीय पादप आनुवंशिक संसाधन ब्यूरो
ICAR – National Bureau of Plant Genetic Resources
A nodal organization in India for the management of plant genetic resources
Germplasm Exchange & Quarantine Information System

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ICAR-National Bureau of Plant Genetic Resources
(भारतीय कृषि अनुसंधान परिषद)
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Pusa Campus, New Delhi - 110 012
Phone/दूरभाष: 25841619 • FAX: 91-11-25842495 • E-mail/ई-मेल nbpgr.exchange@icar.gov.in

TAX INVOICE
(Original Handling Charges and Quarantine Processing Fee Receipt for Recipient)

Invoice No.: IQ-330/2017 **Dated: 03 Feb 2018**

Consignment Handling Charges and Quarantine Processing Fee			
Import Quarantine (IQ) No. 330/2017	**Import Permit No.** 324/2017	**Country** Mexico	**Crop** 150 Seed samples of Wheat
Importer	Dr. T. Venkata Reddy, ITC Limited, ITC Life Sciences & Technology Centre, #3,1st Main Road, Peenya Industrial Area 1st Phase, Bengaluru-560058 (Karnataka) GST No. ITC-LSTC GSTIN: 29AAACI5950L1Z6		
GSTIN: 07AAAAI1830P3ZJ	**SAC Code:** 998114		**Place of Supply:** Delhi

Consignment handling charges @ Rs. 3000.00/- per consignment	Rs. 3000.00/-
Quarantine processing fee @ Rs. 200.00/- per sample(150 Samples)	Rs. 30000.00/-
CGST @ 9 %	Rs. 0/-
SGST @ 9 %	Rs. 0/-
IGST @ 18 %	Rs. 5940/-
Total	**Rs. 38940/-**

VERSION 2.0 (UPDATED ON: 01 FEB 2018)
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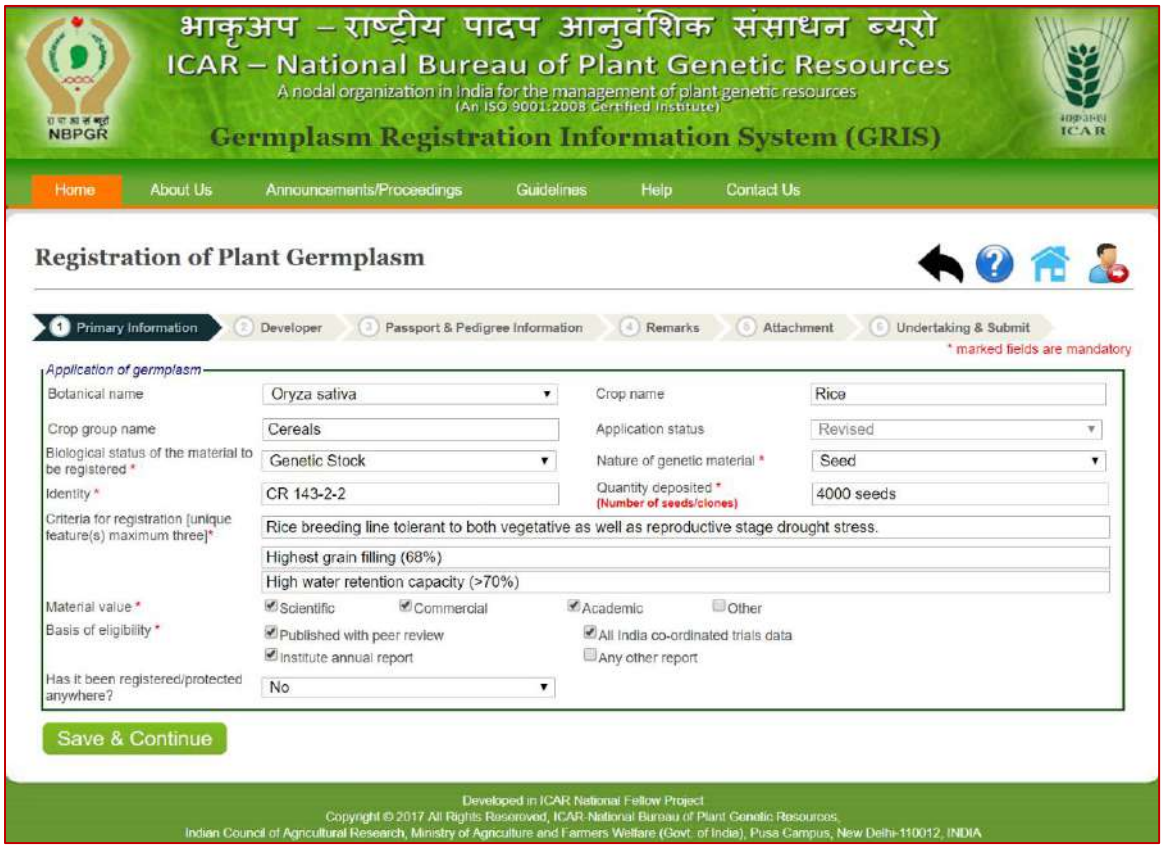
INDIAN COUNCIL OF AGRICULTURAL RESEARCH, MINISTRY OF AGRICULTURE (GOVT. OF INDIA), PUSA CAMPUS, NEW DELHI-110012, INDIA

Germplasm Registration Information System (GRIS)

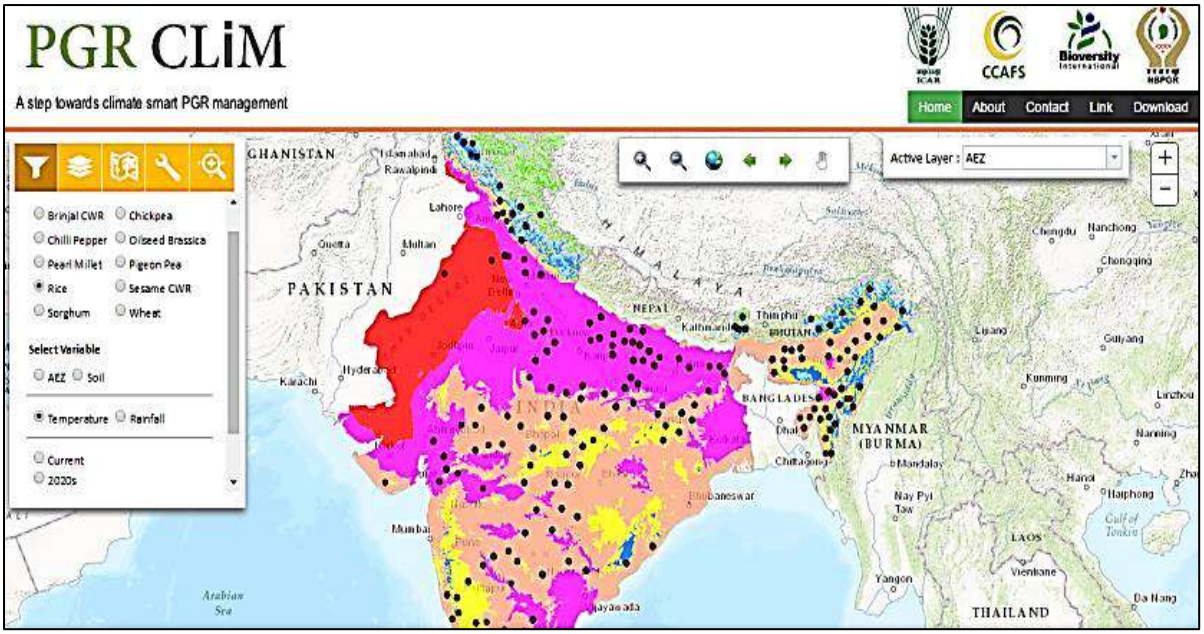
A mechanism for "Registration of Plant Germplasm" was instituted in 1996 by ICAR to serve as a recognized tool for registration of PGRFA at national level facilitated access to the developed or identified potentially valuable germplasm for utilization in crop improvement programs. Since the institution of this mechanism, a total of 1,313 germplasm belonging to 209 crop species have been registered.



Why GRIS: The Germplasm Registration Information System has been developed to make the entire process of germplasm registration—submission of application, evaluation by experts and decision by Plant Germplasm Registration Committee—a web-based system. The system is expected to provide genebank managers, breeders and plant researchers with a hands-on tool for management of germplasm registration process, and to policy makers with a reliable source of information. With the advent of this system, it is expected that the entire process of germplasm registration is made simple and fast, facilitating transparency and expeditious decision making. GRIS also has advantages like real-time tracking of application, speedy disposal, searchability and retrieval of old records.

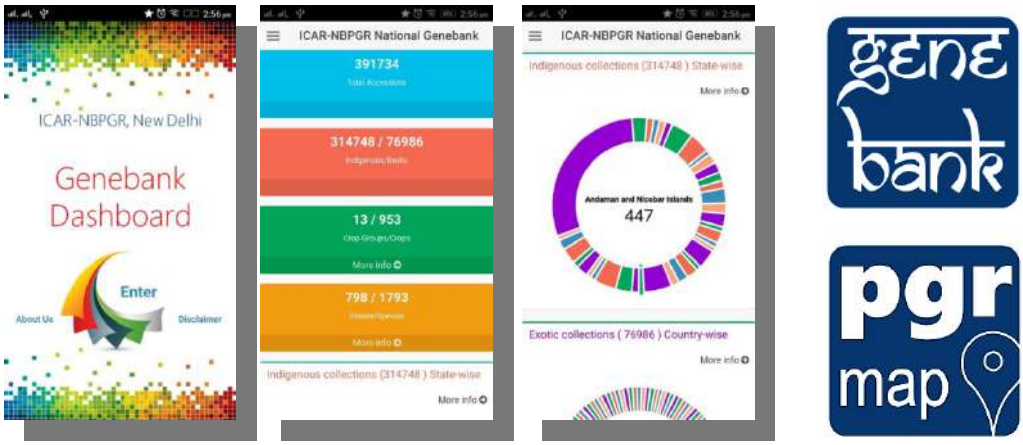
Geo-informatics portal in PGR

A study to link germplasm to changing climatic regimes was earlier carried out with the funding of the CGIAR Research Program on Climate Change, Agriculture and Food Security (CCAFS). A web interface named PGR CLiM was also developed to access information (www.nbpgr.ernet.in:8080/climate). It was improved to be a portal running on a GIS-server that is now interactive to choose layers of germplasm accessions (ten crops); soil type; AEZ; temperature and rainfall (current, 2020 and 2030).



Mobile apps in PGR Informatics

Two mobile apps "Genebank" and "PGR Map" have been developed to enhance access to PGR information with an easy user interface. The apps have been developed for both Android and iOS. The apps have been hosted on Google Play and App Store.



PGR-IP management system

Based on the application reported in 2016, a mobile app, iP-PGR, was developed for easy access to information. Application development was supported by ICAR-National Fellow project. iP-PGR can be downloaded from google play store.

Conclusions

In spite of the above mentioned progress, Indian efforts in PGR informatics are yet to achieve progress in many vital areas for the following reasons: (i) ICAR-NBPGR conserves all crop species; (ii) NBPGR carries out all activities of PGR management and hence has to manage multiple types of data (Passport, Genebank, Characterization, Exchange, Quarantine, DNA fingerprints, etc.); (iii) Connecting more people requires development of multi-lingual applications.

Acknowledgements

PGR Informatics products and services at NBPGR are a culmination of efforts and support of several individuals of the Bureau over a long period of time. PGR Databases were designed and developed with support of ICAR through institutional funding. Development of web-based and mobile applications was supported by ICAR-National Fellow Project principally

on the basis of recommendations of the erstwhile National Board for the Management of Genetic Resources.

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Visit *pgrinformatics.nbpgr.ernet.in* to learn more about PGR Informatics applications

18

From Field to the Herbarium of Cultivated Plants "The Hidden Garden of Plant Genetic Resources"

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The cultivated plant herbarium at the ICAR-NBPGR is a hidden garden of plant genetic resources where diversity brought from field is preserved and used for the benefit of users. This chapter includes the steps from collection of herbarium specimens to preparation and inclusion of the same in the NHCP (National Herbarium of Cultivated Plants). In view of the difficulty in representing the material of cultivated plant genetic resources, the steps from field to herbarium are discussed here.

Plant species growing in the fields can be traced back in a herbarium in the form of dried specimens with authentic records of its place of occurrence. The herbarium is the repository of unique resources for the genetic resource programme. The herbarium (a repository of dried plant specimens identified and grouped on the basis of similarities using a standard system of classification to serves as valuable resources for plant identification and data used for scientific research) are prepared using standard procedures. An ideal herbarium specimen should represent all plant parts and information on locality, habitat and plant characters of collected material (Figure 1). The information on plant species about area of availability, variability pattern, flowering/fruiting time, status regarding rare/endangered/endemic types, economic uses, *etc.* gathered from herbarium data may help the users in various ways.

Field studies are undertaken in Plant Genetic Resource (PGR) management including the floristic surveys and germplasm collections. While moving in field the knowledge on species/taxon is one of the basic requirements. Planning of field visits for collection of the herbarium specimen starts with the visit to the herbaria and online databases which should be referred for gathering data on distribution pattern, localities, diversity pattern and period of collection particularly for wild species.

Areas of visit are selected based on the objectives of the herbarium collection of an unrepresented taxa. Agricultural fields are the best areas for collection of crops as well as wild/ weedy plants with extent of diversity. While proceeding on field visits, the collector has to be well equipped with tools and material essential for conducting trips (refer to Annexure I). Regional/ national flora, digital herbarium, list of local names of plants, road-map, vegetation/climate map, list of rest-houses/ lodges, hotels, resting/ stay places and list of local contacts (phone, fax, e-mail) are essentials items required during field visits.

Hebarium specimen preparation needs collection of samples at full bloom stage whereas for fruit/seeds collecting full physiological maturity of germplasm is needed. In a single trip, the collector may find difficulty in assembling the germplasm and herbarium both; two trips may be planned by the same team or else two different teams can visit with different objectives. Field photography is an essential tool to facilitate capturing the entire picture of the area along with plant diversity. Closer-view of plants/parts when captured may be helpful in identification of characters which otherwise upon preparing a herbarium may be lost. The detailed guidelines (Jain and Rao 1977) for preparation and processing herbarium specimen should be followed. The major steps involved in collection, processing and inclusion of the specimens in the herbarium are as follows:

Collection and processing

During the journey of plant species to the herbarium the collected sample goes through the hands of a plant collectors (botanists, explorers, researchers, *etc.*) who visits the field, garden, or net house/greenhouse, and selecting a representative specimen. Plants must be carefully uprooted to ensure that the genetic diversity is retained on-site and not destroyed. Sometimes biased sampling can be done of the selected type. Using a strong knife, a pair of pruning shears or secateurs, plant is dug out to take the underground parts. Polythene bags or vascula (metal collecting cans) are also used to collect the material. While collecting herbarium specimen formaldehyde (about 1 per cent) may be spread to avoid any infection especially with bulky fruits, cucurbit material. Delicate plants that tend to wilt fast may be collected and pressed right in the field using thick blotters. A tag with collector number, date of collection, is attached to individual plants while collecting. Latitude-longitude, soil, temperature, rainfall data should be recorded at the site of collection. The collector notes on details of the characters which are lost on drying, or which may not be represented in the herbarium specimen (plant height), flower colour, leaflets (which may be shed on drying) should be mentioned in field notes.

Normally the minimum number of herbarium specimens in a collection should be three-five but if the plants are small an attempt should be made to collect several (at least 5-6) specimens of the same plant from the same locality. The specimen should consist of whole plants complete with roots, stem, leaves, flowers and if possible fruits. Where the plants are shrubs or trees, it is necessary to select a portion as a representative specimen the size of which is normally determined by the size of the mounting sheet. In case of dioecious plants try to collect both male and female plants/ branches (to be given different number). When flowers and fruits are too large to be pressed with the leaves, they are processed separately or if can be dried (especially woody fruits like *Feronia limonia i.e.* wood apple) and stored in wet collection or in boxes. Extra specimens of the same collection can be made as duplicates for exchange/studies. All additional notes as collector's name and collection number, place and date of collection, and features of the plant not shown by the dried specimen as soil types, associated flora, distribution, *etc.* should be recorded in the field note book (Annexure

1). Type of material: Depending on the objective of the collection mission, seed, vegetative propagule, *in vitro* material and pollen are collected. The herbarium specimens in general and especially of the wild types and wild relatives should be collected to help in identification/ authentication. Efforts should be made to collect economic products of local/specific use for potential/ lesser know group as supportive samples.

Individual specimen is neatly arranged with all plant parts well spread using blotters or newspaper sheets. Number of sheets may depend on type of material, for examples the cereals generally can be easily taken care while the vegetables especially the ones with fleshy material need many sheets to avoid fungal growth during initial processing phase. Similarly it also depends on the season when plants are dried. Generally during wet season much care with more sheets can be put for good results. Care should be taken to avoid overlapping of plant parts; corrugated sheets are placed between the blotters and the specimen if processing done using artificial drying method or during wet season. The whole bundle is tied using a strong belt/strap and placed in a plant press.

Cultivated plants have comparatively fleshy parts especially the fruit plants (cucurbits, solanaceous fruits), those with large leaves/ flower buds (cauliflower, cabbage) and therefore difficult to press as herbarium specimen. There is choice to represent them in wet collection (4 per cent formalin) or in died form/ cut section so as not to miss out characters during drying. Putting them as wet collection has major disadvantages that they occupy large space, rose-colored are also difficult to handle. While representing herbarium specimens of crop cultivars, large/rhizomatous/tuberous/bulbous types, aquatic/ fleshy material and other problem taxa reference should be made to Pandey (2019).

Drying methods

The herbarium press containing specimens is left tied for 24 hours (sweating period) and on first opening, all plant parts are neatly rearranged on fresh blotters. The bundle of specimens is again put in the press for 24-36 hours. Same process is repeated for about a week or until perfect drying is complete (specimen stops limping); used blotters may be recycled after drying. Plant specimens are usually processed right in the field or at least the same day. If left overnight they tend to deteriorate. In general the thick or succulent material requires more time and special care should be taken to avoid any kind of infestation/ deterioration.

In an alternative method, specimens after processing for 24 hours in field press are rearranged and placed over a heat source (drying chambers, ovens, stoves, *etc.*). The temperature is adjusted to 46-50^{o}C. The hot air passing through the corrugated sheets (placed between the blotters) facilitates drying of the specimens. Time taken for drying varies and this procedure may be modified based on type of material. Special technique are adopted using micro-wave oven for succulents or fleshy material such as fleshy roots in vegetables, big sized fruits as in cucurbits or large leaved plants like aroids roots/ tuberous material. For each atypical specimen a modified protocol needs to be developed for effective drying.

Preservation

The specimen preservation at collection stage is done by poisoning immediately after collection or at the time of mounting using chemical treatment (alcohol is particularly done to avoid the microbial damage under high humidity conditions). Pouring 10% formalin over specimens contained in the press and placed in an airtight polythene bag facilitates removing any field contaminated infection while in field. Dipping/ spraying with saturated solution of mercuric chloride in ethyl alcohol and processing the same for drying is generally practiced. Preservation of the mounted specimens needs application of chemicals through a brush may be used. Under storage the specimens are fumigated when infestation of pests is high. Volatile poisonous liquids like carbon disulphide, methyl bromide, carbon tetrachloride are also used sometimes. Specimens should remain airtight (for 3-4 days) to efficiently work and avoid the health hazards.

A routine practice of deep freezing subjected to a period of 72 hours (at -20^{o}C) has been found more effective in controlling the pests/pathogens during processing or under storage. This method is also used for new material received from outside the herbarium. Sometimes in case of mild infection by storage pests, the dusting of specimens with naphthalene powder is also practiced.

Mounting of specimens

Completely dried, poisoned specimens are mounted on good quality, standard-sized mounting sheets/boards (40x28cm). Different materials such as glue, paste, narrow strips of glued linen, a needle and thread, *etc*. are used for mounting. The glue or paste is usually applied using a brush. An alternative procedure is to spread the glue over a sheet of glass over which the lower side of the plant is placed before mounting on sheet. Stiff/bulky plant parts are usually tied using needle and thread. The herbarium label (11x6.5cm) containing information on plant name, family, local name, date of collection, place of collection, collector number, status (flowering/vegetative) should be pasted on the bottom right hand corner with information typed or filled with permanent ink. Herbarium label gives basic information on the accepted name of specimen when collected from its natural habitat, the location, date of collection, collector's name, identity etc. (Annexure 1).

Identification of the plant specimen

The process of identification is done before the mounting of specimen on board or after mounting depending on whether the specimen is to be identified in the herbarium or sent to the expert. Among the plant characters, the floral and fruit characters have been found of much relevance in taxonomy of cultivated plants (Davis and Heywood 1963).Identification methods involve study of the plant characters, careful examination and comparison of the characters *vis-a-vis* the description in the regional floras using family, genus and species keys, and cross matching with already available identified specimens. When no clues are available plants from adjacent regions are looked at and reference is made to larger herbaria.

In crop plants normally well known taxa are easily recognized but some time less-known species and crop wild relatives/wild economic plants may demand extra efforts. For correct identification help may be sought from the experts. Annotation or determinavit (det.) slip (small slip attached on the herbarium sheet) should put on the herbarium board to indicate name changes/correct identity of a plant by person annotating with his name and signatures with date alongwith institutional affiliation.

Establishing taxonomic identity

Material with unknown identity, or identity only with vernacular name should be collected along with herbarium specimen and photographs for identification/authentication. When a collector is unable to collect herbarium specimens, efforts should be made to retain fruits, flowers and leaves along with seeds, and also to raise plants in grow out to establish its correct identity.

Most floras begin with a key to the plant families, use of which for identification may be a cumbersome process. Thus, knowing the general characteristics of families helps in proceeding directly to the key of genera within that family. Practical steps taken for identification of plant taxa often depend on the experience of identifier/determiner. Similarly, to get an idea as to the general group within a family to which the taxon belongs (*e.g.,* a suspected genus), keys, illustrations, descriptions, within the group they can be consulted.

The process of identification necessitates describing the plant in question with complete morphological characterization. One should check the geographic range of the flora used. Cultivated plants can be particularly difficult to identify. As the number of plants taken into cultivation is quite large and may also be difficult to identify as they are continually being evolved from an original native species (variant/cultivars).

Among the most popular methods used for identifying plants the keys are probably the most popular, practical and utilized method of identification. Taxonomic keys are of two types: dichotomous and polyclave (also called multiple access or synoptic). Dichotomous keys are also called 'forked keys' are the most common type, consisting of a series of paired statements, termed *couplets* that describe some feature of the organism. The statements, or *leads*, are in direct contrast (*i.e.,* mutually exclusive). To use the key, begin with the first couplet and select the statement that best fits your specimen. This will direct you to another couplet and ultimately provide the identity of your specimen. There are two types of dichotomous keys viz. indented keys and bracketed keys. Polyclave key consists of a list of numerous character states, whereby the user selects all of states that match the specimen.

Written description: this is a good method of determining with certainty whether the range of variation of the unknown plant corresponds to that listed in the description of a known plant. This method relies on narrowing down the possibilities first. An identifier has to compare features of the unknown plant with written descriptions of the possible known taxa. Thus,

written descriptions are the best used to verify an identity after one or a few possibilities are worked out.

Specimen comparison

Use of an identified herbarium specimen comparison to verifying an known sets the possibilities to narrowed down. Herbarium collections, which house generally one specimen of each taxon for a given region (e.g., a state, district, county) are very useful in this regard. If a taxon can be narrowed to a smaller group, such as a family or genus, a quick search through a synoptic collection for that region may often allow for site identification of the unknown.

Image comparison

An unknown plant may be identified by visually comparing it to photographs or illustrations of known taxa. These are usually obtained from books, although webpage images have now become a very useful resource. A practical problem with this method is that photographs and illustrations are usually available only for limited taxa. However, visual comparison to an image can still be an excellent way to identify a plant but the major precaution about this method is that two or more taxa may look very similar and the differences between them are not captured.

Expert determination

This method of seeking help, preferably of an expert in the group in question, requires sending a specimen for identification. Expert determination is often essential for certain groups in which species or infraspecific identification is very difficult. After the entire process, one should not over rule the possibility that indicates that the unknown plant in question may be a new record (either native or naturalized) for the geographic range of that flora.

Indexing, documentation and housing of herbarium specimens

After mounting, labeling and identification, specimens are given a Herbarium Accession Number (unique index number), then the herbarium specimens are arranged in a hierarchical system: Species - genera - family - order and so on; a standard system of classification such as that of Bentham and Hooker, or Engler & Prantl (Lawrence, 1951) is used to organize collection.

Generally the herbaria have separate section for housing the 'Type specimens'. The 'Type specimens' [(herbarium specimen(s) prepared and used by the author for describing a taxon/ species] are housed separately with special care and are not handled routinely. A system of different coloured genus folders for different geographic regions can be used to facilitate quick review of general distribution of a species. Duplicate collections are numbered (same as original collection but placed separately for use in exchange or other study.

In modern herbaria, digital herbarium (also called e-herbarium or virtual herbarium) are a part of the main herbarium. It is essentially a collection of high resolution digital images of a traditional herbarium sheets. Additional information about each specimen, such as the passport data and taxonomic identity, is attached to every specimen.

Initiation of digitization of herbarium collections started with larger herbaria long back for the purpose of maintaining their own database and web site. But due to gradual declining interest of the scientists/technical towards taxonomy and many technical problems in herbarium management (labour, funds, and staff) the institutions housing herbaria are giving low priority to related activities. The digitizing process consists of predigitization curation, imaging specimens, databasing and identifying information, and dereferencing locality information. Digitizing process required six steps: (1) organizing all specimens in the cabinets using updated taxonomy and nomenclature including many new collections, (2) setting up an imaging station, (3) developing efficient imaging protocols, imaging each specimen individually, (4) choosing the database template to fit to our need, (5) updating card/label information and entering in the database, and (6) collating all data with images, and (7) exporting to web through an online database for use.

At the herbarium, many specimens lack the data on exact location of collection sites noted on the label. In such cases data can be used to approximate GPS coordinates using georeferencing software. NHCP has initiated to the georeferencing of herbarium specimen of the selected taxa and intends to continue.

Imaging and data storage is done for each specimen developing efficient imaging protocols. Screen shots Images were captured in JPEG. Imaging area was identified along the cabinets and computer workstation so as to shorten the time of taking out the specimens to be digitized and also non-mixing and misplacement of the specimen due to in out process. The capture data (images and data sheets) are stored at least two backups in different locations, as well as in an external hard drive is needed to store them. Having a barcoding system and globally unique identifiers (GUIDs) can help prevent duplicated records. Images may represent a significant amount of data and are accessible to the scientific community and public through an online database.

Herbarium specimens represented in the National Herbarium of Cultivated Plants (NHCP): The NHCP at the National Bureau of Plant Genetic Resources (NBPGR), New Delhi is intended to serve as a reference collection for identification purposes, and taxonomic study of taxa of PGR relevance. It differs in its mandate from the existing national herbaria in that emphasis is laid on the collection of the wide range of variability available in crop plants in different agro-ecological situations in the country and from exotic sources and also generated through breeding and selection. Equal emphasis is also laid on the collection and deposition of the vouchers of wild relatives and related types; cultivars, primitive landraces, *etc.* of the material /collections under PGR programmes, medicinal plants of economic and

potential value. The experimental material the voucher samples are deposited at the end of specific researches or specimens received for identification.

The NHCP, presently, has 23,942 herbarium specimens representative of 267 families, 1526 genera and 4299 species (as on August 31, 2019). The build-up of herbarium specimens and supplementary material is through specimens/ seeds collected during explorations to different agro-ecological zones of India and also material introduced from exotic sources and raised as plants and also the vouchers of the systematic studies of crop groups (Figure 1).

Figure 1: Herbarium processing takes many steps for inclusion.

The digital images are a part of virtual herbarium and are provided for use as a database through the AKMU (Agricultural Knowledge Management Unit) of the ICAR-NBPGR. This can be accessed through URL http://pgrinformatics.nbpgr.ernet.in/nhcp. The web-based interactive NHCP aims to provide access to the valuable information on identities of over 2,000 specimens of genetic resource value including exotic taxa and the crop wild relatives. The database would be available to a wider user base subsequently of the ICAR-NBPGR (to be continued) to make the data available to a wide users.

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Annexure I

Herbarium Record, NHCP, NBPGR, New Delhi

Botanical Name

Family

Local Name

Loc. (Place, Town, Dist. State)

Date of Collection

Collector's Name and No.

Field Collection/Grown in experimental Conditions:

Identified by

Nature of Specimen (Adult Plant /Seedling, Vegetative/Flowering/Fruiting etc.)

No. of Specimens

Additional material supplied (Seed, fruit, Economic Product) users, if any, in locality of collection

Notes

Herb. specimen No.: 1 2 3 4

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Detection and Identification of Pests in Exotic Germplasm including Transgenics

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Detection and identification of pests are crucial for safe exchange of germplasm including transgenics. The present day definition of a pest is any species, strain or biotype of plant, animal or pathogenic agent injurious to plants or plant products. A quarantine pest is, the pest of potential economic importance to the area endangered thereby and not yet present there, or present but not widely distributed, and being officially controlled (http://www.ippc.org). The successful detection of pests in seed and other planting material depend upon the availability of rapid, reliable, robust, specific and sensitive methods for detection and identification of pests. Over the years a great variety of methods have been developed that permits the detection and identification of pests.

At ICAR-NBPGR, a team of experts from Pathology (Fungi, Bacteria and Viruses), Entomology, Nematology and Agronomy disciplines examine the samples for presence/absence of pests. The actual quarantine processing starts with the examination of material by naked eye or with the help of magnifying glass as well as examination under stereo-binocular microscope for detecting the presence of discoloured, deformed seeds, bunt balls/ spores, ergot sclerotia, rust pustules/ spores, crust of downy mildew/ spores, presence of yellow discolouration around the hilum, insect damaged seeds, flour, dead or actively moving larval and adult stages, webbing, presence of excreta, soil clods, nematode galls, plant debris, weed seeds, etc.

Specialized Detection of Pests: Samples are thereafter processed through various specialized techniques for each discipline. On detection of a pest the material is either salvaged using different disinfection/disinfestations techniques or rejected depending upon the category pest detected. The methodologies adopted for detection of different pests are given in sections I-V presented below:

SECTION I: Detection and Identification of Plant Viruses

V Celia Chalam and AK Maurya

The successful detection and control of viruses in seed and other planting material depend

upon the availability of rapid, reliable, robust, specific and sensitive methods for detection and identification of viruses. Virus indexing is done by deploying a combination of certain well known virus detection techniques keeping in view the intended purpose. These are enumerated below in brief:

Visual Examination of Planting Material: This is often done with the help of an illuminating magnifier. A variety of symptoms on seed have been associated with virus infection. Symptoms associated with virus infection are mottling on soybean seed coat due to *Soybean mosaic virus*; split seed coat and necrotic line pattern on seed coat of pea due to *Pea seed borne mosaic virus*; necrotic lines on the seeds of broad bean due to *Broad bean stain virus*, split seed coat in green gram due to *Bean common mosaic virus*, etc. (Fig. 1) The seeds free from seed symptoms should be handled for sowing/ exchange/ conservation and removal of seeds with seed symptoms constitutes elimination of potential source of virus inoculum.

Mottling on soybean | Mottling on soybean | Necrosi on faba bean | Split seed coat on green gram | Split seed coat on pea | Tennis ball-like symptom on Pea

Figure 1: Virus symptoms on seeds

Growing-on Test in Post-entry Quarantine Greenhouse: This is based on the symptoms appeared on the young growing seedlings of first trifoliate stage to flowering stage. Seeds treated with Mancozeb + Bavistin (2:1) @ 2g/ Kg should be sown in plastic pots filled with a mixture of sterilized soil and farmyard manure (2:1) or in soil beds of post-entry quarantine greenhouse. The temperature should be maintained at 28±2°C. The seedlings should be observed regularly for symptoms expression (Figure 2) and the percentage of seed transmission of a seed lot should be calculated as given below:

$$\text{Seed transmission (\%)} = \frac{\text{Number of seedlings showing symptoms}}{\text{Total number of seedlings tested}} \times 100$$

Puckering | **Leaf rolling** | **Vein necrosis** | **Mosaic**

Figure 2: Symptoms of plant viruses

Mechanical/ Sap Inoculation: The transmission of a virus from infected to healthy tissues is a procedure fundamental to a study of virus diseases. Mechanical transmission is basically manual transfer and deposition of biologically active virus to a suitable site in the living cell by sub-lethal wounding or abrasion. This may be achieved by inoculating infectious sap on healthy host directly. This procedure is called mechanical or sap transmission and is given below:

- Symptomatic young leaves should be collected from diseased source. In the laboratory such leaves are washed and gently blotted dry with the tissue/blotting paper and their weight is taken.
- Extracts should be prepared by macerating leaves showing severe virus-like symptoms in 0.2 M phosphate buffer pH 7.2 (1:10 w/ v) using chilled (0^0 C) mortar and pestle. The extract should be filtered using absorbent cotton.
- Add celite/ carborundum to the filtered extract or lightly dust celite/ carborundum on the leaves of test plants.
- Cotton pad soaked in standard extract is rubbed on the leaves of test plants (supporting the leaf from the central surface with a palm) in one direction only i.e. from the petiole to the apex of the leaf.
- After inoculation, plants are washed with water to remove excessive inocula and extraneous particles (for this purpose a wash bottle is used) and keep plants in the green house for observation.
- Observe the seedlings daily for the expression of symptoms.

Electron Microscopy: The Transmission Electron Microscope (TEM) can be used directly to detect the presence of virus in the plant tissue. It reveals the shape and size of the virus particle. The shape and size of the virus particle gives an idea of the group to which it may belong. The protocol for leaf dip preparation is given below:

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

- Observe the grid under Transmission Electron Microscope (TEM) for virus particles (Figure 3).

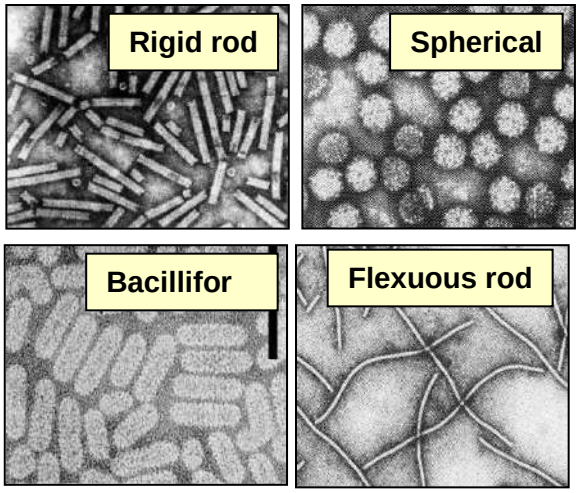


Figure 3: Different virus particles under Transmission Electron Microscope

Double Antibody Sandwich – Enzyme-linked Immunosorbent Assay (DAS-ELISA): The double-antibody sandwich (DAS)-ELISA is a direct ELISA and was the first ELISA procedure developed for plant virus detection. It is still widely used. The protocol is given below:

Sample Preparation

Seeds: Soak seeds in distilled water overnight and grind the seeds in PBS-T buffer, pH 7.4 + 2% polyvinyl pyrrolidone. The extracts (1:20 w:v) should be centrifuged for 3 min. at 3000 rpm and collect the supernatant. Samples will be further processed by DAS-ELISA.

Leaves: Take 0.1g of symptomatic/ healthy-looking leaf sample in a mortar and grind in Phosphate Buffer Saline - Tween 20 (PBS-T) buffer, pH 7.4 + 2% polyvinyl pyrrolidone. The extracts (1:10 w:v) should be centrifuged for 3 min. at 3000 rpm and collect the supernatant. Samples will be further processed by DAS-ELISA.

Buffers used for DAS-ELISA

1. Carbonate Buffer pH 9.6

Na_2CO_3	1.59 g
$NaHCO_3$	2.93 g

Dissolve in distilled water and dilute to make 1L

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

NaCl	8.0 g
Na_2HPO_4 12 H_2O	2.9 g
KH_2PO_4	0.2 g

KCl	0.2 g
Tween 20	0.5 ml

Dissolve in distilled water and dilute to make 1L

3. Sample Extraction Buffer pH 7.4

Polyvinyl pyrrolidone	20 g
NaN_3	0.1g
Dissolve in PBST and dilute to make	1L

4. Conjugate Buffer pH 7.4

Polyvinyl pyrrolidone	20 g
Bovine serum albumin	2 g
NaN_3	0.1 g
Dissolve in PBST and dilute to make	1L

5. Substrate Buffer pH 9.8

Diethanolamine	97 ml

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

6. Substrate Solution *para*-nitro phenyl phosphate (pNPP)1 mg/ ml in substrate buffer

Protocol Followed for DAS-ELISA

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

↓

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

↓

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

↓

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

↓

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

↓

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

Yellow colour is developed in samples infected by virus. Consider the sample as infected if its OD value is twice the mean of negative control (Figure 4).

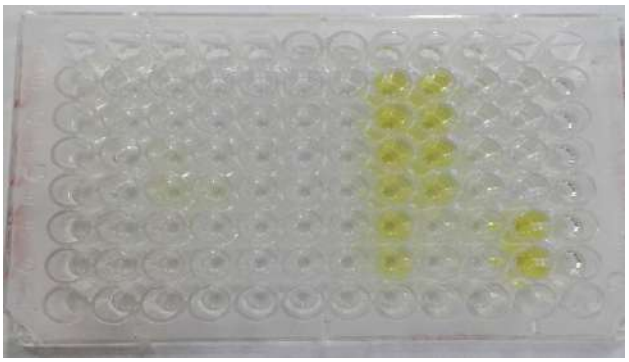


Figure 4: DAS-ELISA of French Bean Seed Samples against *Cherry leaf roll virus*

Lateral Flow Strip Method: Lateral flow strip method is a variation of ELISA used for detecting viruses, and the antibodies are immobilised onto a test strip in specific zones. The extracts from seed/ leaf are prepared and lateral flow strips are dipped into the extract and results are obtained in 15 to 20 min. The appearance of two lines on the strip indicate that the sample is positive.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR): Most of the plant viruses consists of RNA, which require the introduction of a preliminary reverse transcription (RT) step before the PCR amplification process (RT-PCR), thus allowing the amplification of RNA sequences in a cDNA form. Many viruses have been detected using RT-PCR (Chalam and Khetarpal, 2008; Chalam *et al*., 2004, 2012). The methodology is given below:

- Extract total nucleic acids from symptomatic and healthy-looking leaves/ seeds
- Synthesise first strand (cDNA)

  10 µl DNA/ RNA mix (out of 60 µl)

  8 µl 5X 1st St buffer

  8 µl 2.5 mM bases

  4 µl 0.1 M DTT

  0.5 µl 1st primer (200 µM stock)

  8.0 µl H_2O

  65°C 3 minutes quick ice/ water bath

  0.5 µl Rnase out

  1 µl MMLV Reverse Transcriptase

  Incubate at 42°C 1-2 hrs
- Keep the reaction in thermal cycler
- Mix the following in order to a sterile 0.5 ml microfuge tube.
  2 µl 1:5 dilution 1st strand reaction
  2 µl 10 x PCR

1.2 µl 2.5 mM bases
0.8 µl 50 mM $MgCl_2$
0.6 µl 20 µM primer 1 (specific to the virus)
0.6 µl 20 µM primer 2 (specific to the virus)
2.6 µl ddH_20
0.2 µl Taq DNA polymerase
Total volume = 20 µl

- Carry out the amplification as follows in thermal cycler:

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

- The amplified products are analysed by agarose gel electrophoresis.

The band of the DNA fragment at expected base pair length will be observed as prominent fluorescing indicating amplification. Its molecular weight will be determined by comparing with the molecular weight of marker run on the same gel (Figure 5).

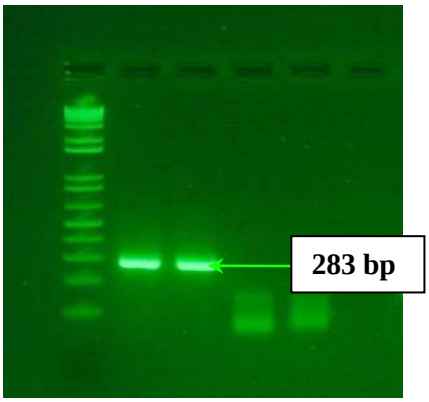


Figure 5: Gel Electrophoretic Analysis of RT-PCR of *Cherry leaf roll virus*

Real Time RT-PCR: Real-time PCR monitors the fluorescence emitted during the reaction as an indicator of amplicon production during each PCR cycle (i.e., in real time) as opposed to the endpoint detection. The real-time progress of the reaction can be viewed. Real-time PCR quantitation eliminates post-PCR processing of PCR products. This helps to increase throughput and reduce the chances of carryover contamination. No-post PCR processing (no electrophoretical separation of amplified DNA) is required. The advantages of Real-time PCR/ Real-time RT-PCR are it is not influenced by non-specific amplification; amplification can be monitored real-time; no post-PCR processing of products (high throughput, low contamination risk); ultra-rapid cycling (30 minutes to 2 hours); require 1000-fold less RNA

than conventional assays and is most specific, sensitive and reproducible. The technique has been successfully exploited for detecting viruses (Chalam *et al*., 2004, 2012).

Loop Mediated Isothermal Amplification: Loop-mediated isothermal amplification (LAMP) is a novel technique that requires only one enzyme having strand displacement activity for amplification under isothermal conditions. LAMP has a higher specificity than PCR because its four primers recognize six distinct regions on the targeted genome. LAMP has been successfully used for detection of *High plains virus* (Arif *et al*., 2012).

Helicase Dependent Amplification: Helicase dependant amplification (HDA), requires no thermocycler for enhanced isothermal DNA amplification and has been successfully used for detection of *Bean pod mottle virus* (Chalam *et al*., 2012).

Important Viruses Intercepted

Adopting a workable strategy using a combination of virus detection techniques viz., growing-on test, infectivity test, electron microscopy, variants of ELISA viz., DAC-ELISA and DAS-ELISA, and RT-PCR, 45 viruses which are either not known to occur in India or are known to possess virulent strains or not known to occur in India on particular host(s), have been intercepted in germplasm including transgenics imported from many countries including Consultative Group on International Agricultural Research (CGIAR) Centres. The interception includes 19 destructive viruses yet not reported from India viz., viz., *Barley stripe mosaic virus*, *Bean mild mosaic virus*, *Bean pod mottle virus*, *Broad bean mottle virus*, *Broad bean stain virus*, *Broad bean true mosaic virus*, *Cherry leaf roll virus*, *Cowpea mottle virus*, *Cowpea severe mosaic virus*, Dioscorea latent virus, *Garlic virus C*, High plains virus, *Maize chlorotic mottle virus*, *Pea enation mosaic virus*, *Peanut sunt virus*, *Pepino mosaic virus*, *Raspberry ringspot virus*, *Tomato ringspot virus* and *Wheat streak mosaic virus*. Besides, 21 viruses that were not reported to occur on the hosts in India were also intercepted and hence they are of quarantine significance (Chalam and Khetarpal, 2008; Chalam *et al*., 2017). If not intercepted, some of the above quarantine viruses could have been introduced into our agricultural fields and caused havoc to our productions. In all these cases the harvest from virus- free plants was only used for further distribution and conservation. Thus eliminated the introduction of exotic viruses.

Conclusion

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

SECTION II: Detection and Identification of Fungi and Bacteria

Jameel Akhtar, Raj Kiran, Pardeep Kumar, Meena Shekhar and SC Dubey

The Division of Plant Quarantine at ICAR-NBPGR has developed procedures for systematic and stepwise processing for detection of pathogens associated with exotic germplasm (Figure 1). Seed-borne pathogens may result in poor quality seed, loss in germination, development of epiphytotics, distribution of new strains or physiological races of pathogens along with the seeds and planting material to new geographical areas. Therefore, critical examinations with specialized testing methods are conducted to ensure pathogens-free import of seeds and other planting materials. The methods used for quarantine testing are:

(i) Visual examination
(ii) Washing test
(iii) Seed soaking method
(iv) Incubation methods (Blotter/ Agar plate)
(v) Molecular detection

Visual examination of seeds: Preliminary examination with naked eye or with the help of a magnifier helps detecting presence of abnormalities such as discoloration, deformation shriveling, pigmentation, malformation of seed with fungal growth like mycelial mats or fructifications like chlamydospores, acervuli, pycnidia, perithecia and other impurities associated with a seed lot such as sclerotia, smut balls, or spore masses, soil clods, plant debris, etc. (Mathur and Kongsdal, 2003). Visual and stereo-binocular examination of seeds results in detection of exotic pathogens in imported crop germplasm. Some fungal disease symptoms such as ergot of wheat (*Claviceps purpurea*), Karnal bunt (*Tilletia indica*) and hill bunt (*T. carries/ T. foetida*) of wheat; kernel smut (*T. barclayana*) of rice; downy mildew (*Peronospora manshurica*) of soybean, etc. (Figure 2).

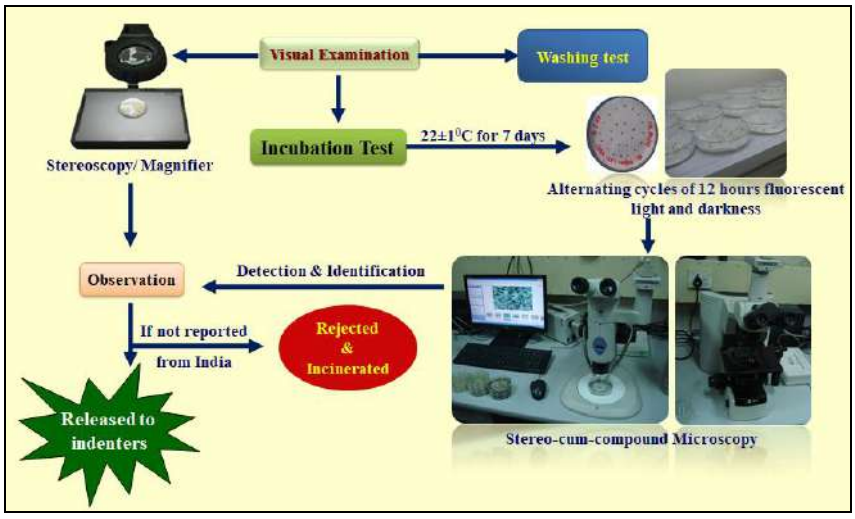


Figure 1: Quarantine testing procedure for pathogen-free release of exotic crop germplasm

a **b** **c** **d** **e**

Figure 2: Symptoms of seed-borne fungal diseases in different crops; a) *Claviceps purpurea*; b & c) Karnal bunt and hill bunt of wheat; d) kernel smut of rice; e) downy mildew of soybean

Washing test: The washing test is commonly employed to detect the presence of fungal spores adhering to seed surface. The method is quick and can be employed for detecting various types of fungal spores such as conidia, oospores (downy mildews), teleutospores/ uredospores (rusts), smut/bunt spores, etc. (Mathur and Kongsdal, 2003). For detection of *Puccinia helianthi*/ *P. carthami* in sunflower/ safflower, the seeds are taken in a test tube containing 10 ml water and stirred on shaker. The suspension is transferred in Petri dish and observed after sedimentation for the presence of spores under stereo-binocular microscope (Figure 3).

(a) (b)

Figure 3: Detection of teliospores of sunflower rust (a) and safflower rust (b)

Seed soaking method: For detection of bunt (*Tilletia barclayana*) in rice, seeds are soaked overnight in 0.2 per cent sodium hydroxide and examined. The infected seed shows shiny jet black discolouration (Mathur and Kongsdal, 2003). Infected seeds rupturing in a drop of water, releases a stream of bunt spores (Figure 4).

Figure 4: NaOH soaking procedure for detection of paddy bunt

Incubation Method: Incubation is a simple method commonly used for detection of mycoflora accompanied as mycelium, spores, or fruiting structures capable of growing on the seed during incubation of seed on wet blotter or agar (Mathur and Kongsdal, 2003). Surface sterilization of the seeds, using a 4% NaOCl solution, is carried out before incubation to eliminate fast growing saprophytes, if the seeds are heavily contaminated.

1. Blotter test: Blotter test, generally referred as the standard blotter test, is the most efficient means of detecting a large number of seed-borne fungal pathogens. Visually discoloured, deformed and unhealthy looking/ suspected seed are undergone for blotter test by placing the seeds on 3 layers of moist blotter paper in plastic petriplates with proper labelling and incubated at 22±2ºC under light in alternate cycles of 12 h light and darkness for 7 days and examined on 8th day under stereo-binocular microscope for presence of seed-borne fungi (Mathur and Kongsdal, 2003). The critical microscopic examination enables the observation of pathogens as developed on their hosts *in situ*, undisturbed and in a condition of natural growth. The identification is confirmed up to species level by making slides for examining the structure, size and colour of fruiting bodies/ conidiophores/ conidia under compound microscope at different levels of magnification i.e. 4.0 X to 40.0 X (Akhtar *et al*., 20014). A critical stereo-cum-compound microscopic examinations of seeds on 8th day after incubation results in detection of many seed-borne pathogens (Figure 5). Major detection includes *Fusarium nivale* on wheat, *Phoma lingam*, *Diplodia maydis* and *D. macrospora*, *Phomopsis longicolla*, *Xanthomonas campestris* pv. *campestris*, *Phomopsis helianthi*, *Dendryphion penicillatum*, etc. on different crop germplasm (Akhtar *et al*., 20016, 2017; Singh *et al*., 2018).

2. Agar plate method*:* Like blotter method, agar plate method is also a standard procedure for detection of many seed-borne fungi/ bacteria. Identification of fungi is based on growth and colony characteristics on nutrient mediums like water agar, malt extract agar, potato dextrose agar. Seeds are surface sterilized before plating on the medium and plates are incubated under conditions similar to blotter method. Identification of fungi is based on the macroscopic examination of colony growth pattern and colour. The identification is confirmed by preparing slides and examining under compound microscope for characteristics of mycelium, spore and conidia.

For detection of bacteria, the surface sterilized seeds are placed on nutrient agar medium in Petri plated and incubated at 22–25°C. The bacterial growth will appear on the seed, if infected. Then the associated bacterium is isolated, purified and identified based on morphological, cultural and biochemical tests, *etc*. Several selective / semi-selective media have been developed and used for detection of specific bacteria associated with seeds.

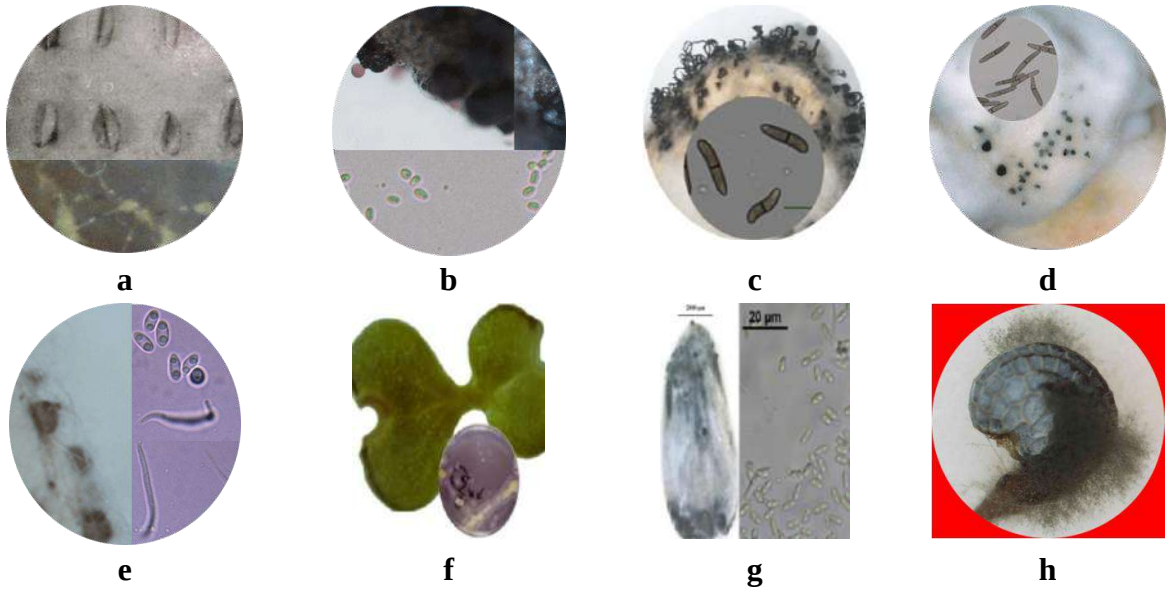


Figure 5: Detection of seed-borne fungal and bacterial pathogens in different crops; a) *Fusarium nivale* on wheat; b) *Phoma lingam* on cabbage; c) *Diplodia maydis* on maize; d) *Diplodia macrospora* on maize; e) *Phomopsis longicolla* on sunflower; f) *Xanthomonas campestris* pv. *campestris* on cabbage; g) *Phomopsis helianthi* on safflower; h) *Dendryphion penicillatum* on opium.

Molecular detection of pathogenic fungi/ bacteria

1. Loop-mediated isothermal amplification based detection of Colletotrichum capsici: A LAMP assay targeting the β-tubulin sequence for visual detection of *C. capsici* was developed, (Kandan *et at*., 2016) The LAMP reaction was optimal at 65°C for 45 min. The results showed that the colour of LAMP products changed from orange to fluorescent green when *C. capsici* was detected with SYBR Green I (Figure 6). All amplified products from *C. capsici* showed bands, while no amplicons were detected for other fungal species and negative control.

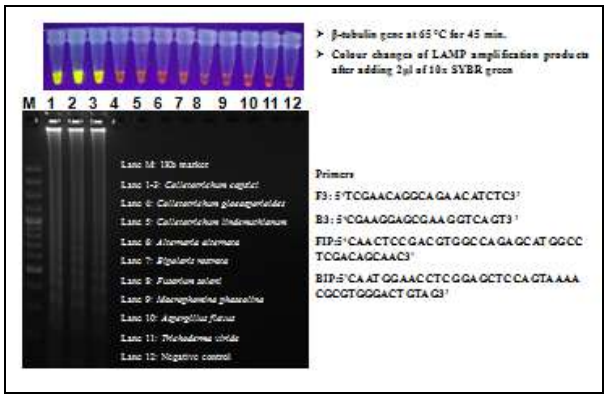


Figure 6: Specificity of LAMP assay for *C. capsici* detection. (left) colour changes of LAMP amplification product after adding 5 ìl 10X SYBR Green I and (right) Agarose gel (1.5%) electrophoresis of 5 ìl LAMP products. M indicates 3 Kb DNA ladder (fermentas), 1 & 2 – *C. capsici*, 3-*Alternaria alternata*, 4-*Bipolaris rostrata*, 5-*Fusarium verticillioides*, 6-*Fusarium solani*, 7-*Macrophomina phaseolina*, 8-*Aspergillus niger* and 9-Negative control.

2. PCR-based detection of Xanthomonas campestris pv. campestris: A set of rpf region-based primers namely rpfH_F and rpfH_R were developed for *Xanthomonas campestris* pv. *campestris.* The annealing temperature for primers were optimized at 60ºC and specific bands of 304 bp for *X. campestris* pv. *campestris* were obtained in PCR (Figure 7). The detection sensitivity of the primer pairs was performed by dilution of genomic DNA and results revealed that it could detect up to 0.1 ng μl^{-1} of template DNA of the pathogen (Kiran *et al*., 2019; Kumar *et al*., 2019). This primer is specific to *X. c.* pv. *campestris* and there is no cross amplification with other related fungal and bacterial pathogens.

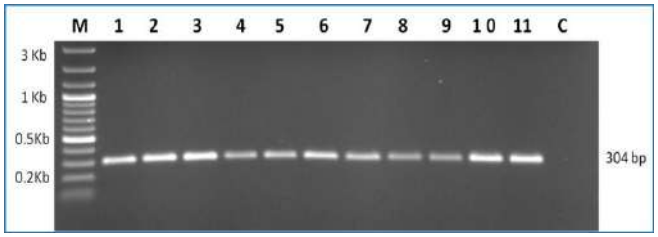


Figure 7: PCR amplification for *X. campestris* pv. *campestris* isolates using specific primers rpfH_F and rpfH_R. Lane M- 1 Kb plus DNA ladder; Lanes 1 to 11- *X. campestris* pv. *campestris* isolates, Lane C- Negative control.

In addition to conventional diagnostic tools, LAMP assay and PCR-based molecular are substantial in detecting and intercepting large number of pathogens of quarantine significance on a wide range of crops from different sources/ countries which emphasizes the pivotal role of plant quarantine in minimizing risk of introduction of exotic pathogens associated with PGR importing into the country, otherwise huge losses would have occurred due to exotic pathogens/ diseases.

SECTION III: Detection and Identification of Plant Parasitic Nematodes

Bharat H Gawade and Zakaullah Khan

Plant parasitic nematodes (PPN) are important parasites of agricultural crops throughout the world with more than 4100 known species. The economic losses caused by nematodes are estimated to the tune of $173 billion (Elling, 2013). Most of the PPN are soil inhabitants while some are aerial parasites. Soil nematodes found in soil around roots of plants. Based on their feeding habit PPN can be are categorized into ecto-parasitic, semi-endoparasitic and endo-parasitic nematodes (Siddiqi, 1986). Majority of PPN directly feed on or in the roots and parasitism by nematodes hampers the uptake and transport of water and nutrients which causes the symptoms like yellowing, stunting and day time wilting. Few species of nematodes like *Anguina tritici, Aphelenchoides besseyi, Aphelenchoides fragariae, Ditylenchus angustus, Diylenchus dipsaci* feed on aerial plant parts.

The nematode spreads from one place to other by active or passive means. The active movement through its own locomotory system is important for plant-to-plant or field to field spread. The passive spread is a long distance movement of nematodes by various agents like wind, water, field equipments, planting material etc. Dispersal through wind, rain and floodwater respects no national boundaries. This kind of spread is important at regional as well as national scale. Many nematodes of quarantine significance as well as of economic importance spread into the new, clean or nematode free areas along with these passive agents, particularly with planting material (Ferris *et al*., 2003). Seed-borne nematodes like *A. tritici, Aphelenchoides arachidis, A. besseyi, D. dipsaci*) have ability to withstand desiccation, which helps in their survival and dispersal. There are other few species, which use insects as vectors for their transmission (*Rhadinaphelenchus cocophilus and Bursaphelenchus xylophilus.* Besides these natural means of dispersal, anthropogenic factors are very common for long distance dispersal of nematodes. Increased trade through land, sea and air tremendously increased over the decades and played very important role in dispersal of devastating nematodes across the boundaries. Exotic nematodes spread through plants, imported grain, plant debris, soil attached to machinery, tyres of vehicles, tools and even shoes. The nematodes like *Pratylenchus penetrans, Hoplolaimus* spp., *Tylenchorhynchus* spp. *Rotylenchus minutus, Meloidogyne* spp. were intercepted from rooted saplings of various plants during processing of exotic germplasm at ICAR-NBPGR, New Delhi. Besides, seeds of paddy are regularly found infested with *A. besseyi.*

In India, examples of introduction of quarantine nematode are golden nematode of potato, *Globodera rostochinesis and Globodera pallida* that spread from Andean mountains to Europe to the India. Burrowing nematode of banana, *Radopholus similis* is another example, the nematode was introduced in India from Sri Lanka, now present in every banana growing region of India and causing heavy losses (Khan, 1999). Similarly, root-knot nematode, *Meloidogyne enterolobii* that was reported recently from Tamil Nadu is causing heavy damage in guava plantations. Therefore, quarantine processing of germplasm for nematode detection and its proper management is utmost important to prevent entry and establishment of new nematodes pests into country. It involves careful observation of germplasm, extraction of nematodes stages, their proper identification and control. Germplasm material received in the quarantine laboratory is subjected to detailed examination by employing various nematode detection techniques depending upon the kind of plant material, size of the sample and availability of time. Some commonly used techniques are described below:

Nematode extraction from suspected germplasm

Seeds / grains*:* Seeds known or suspected to carry seed-borne nematodes are soaked in water which help in softening of seeds. The hard seeds are cut open / teased / crushed which enables the nematodes present in seed to come out in water. The suspension is observed for presence of nematode and if present processed for taxonomic identification.

Plant debris / soil clods: The plant debris or associated soil clods are soaked in water, sieved through nematological sieves of 100, 200, 325 and 400 mesh. The finest sieve (400 mesh per linear inch) allows nematode juveniles and eggs extraction. These are recovered and examined under the compound microscope for identification. Critical examination of accompanying soil is necessary as it carries different stages of nematodes such as eggs, eggmasses, cysts.

Vegetative propagules / roots: The part of the plant tissue especially roots, bulbs, corms, rhizomes etc. is used as a planting material. Migratory endoparasitic nematodes live within root tissue can be extracted using a modified Baermann method. Roots are rinsed, cut into small pieces of 2-3 cm long, macerated using blender with small quantity of water. The incubation for 24 h allows migratory stages to come into water suspension which can be observed under a light microscope. Staining technique is used for quick detection of nematodes are sedentary in nature. The plant tissue is boiled in acid fuchsin lacto-phenol solution for a few minutes and de-stained in clear lacto-phenol. The nematodes, if present, retain the red stain more deeply than the plant tissue and can easily be detected under a stereo microscope.

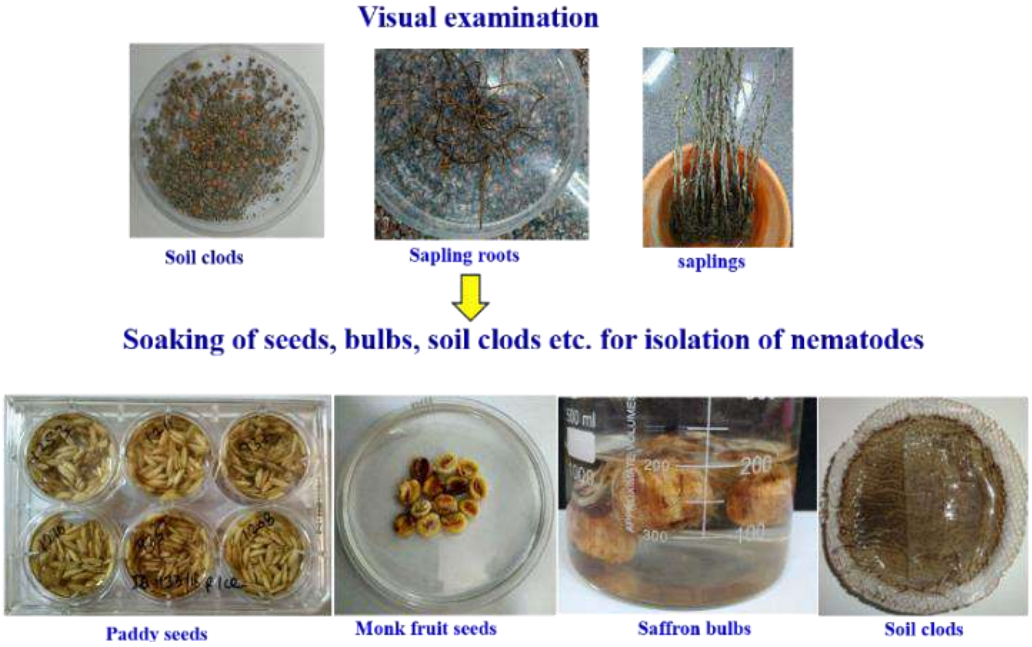


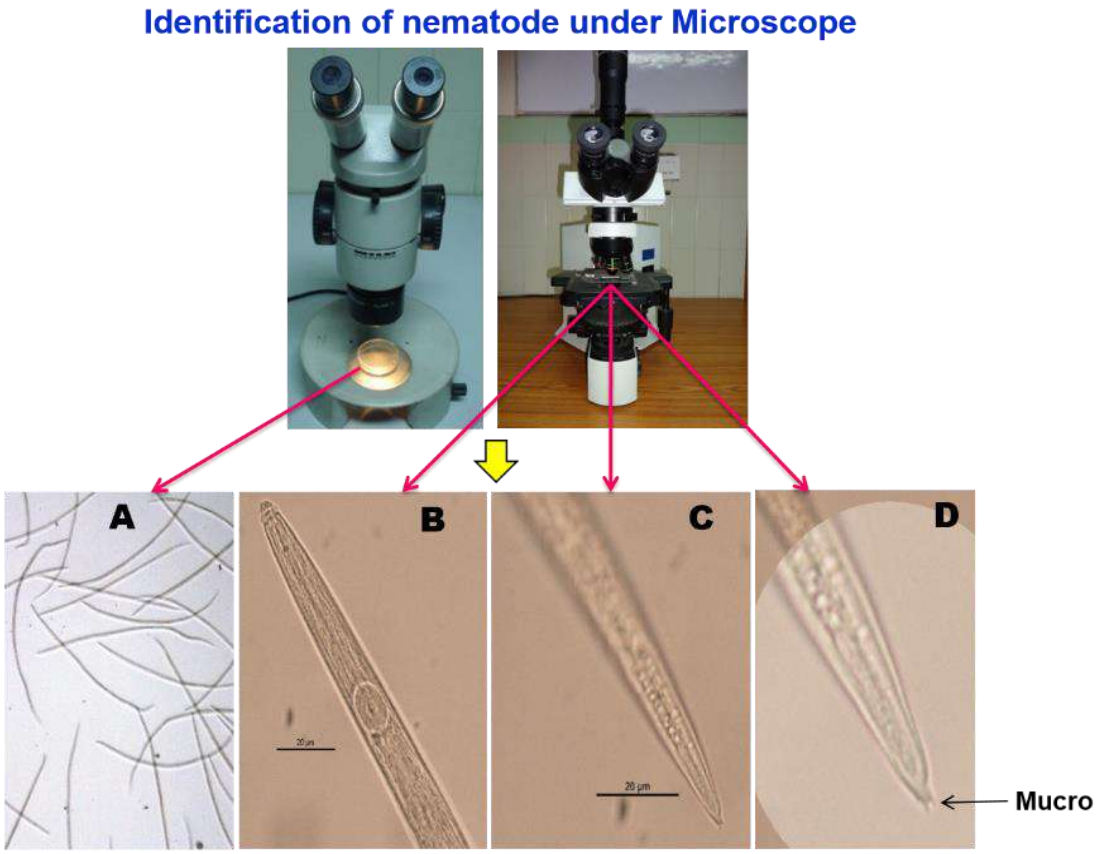
Figure 1: Quarantine processing for nematode detection and identification

Some important nematodes of quarantine importance include *Aphelenchoides besseyi, A. arachidis, Anguina tritici, A. agrostis, Bursaphelenchus cocophilus, B. xylophilus, Ditylenchus africanus, D. destructor, D. dipsaci* and *Heterodera goettingiana.*

Identification of nematodes

The nematodes detected during quarantine processing of samples are identified using morphological, biochemical and molecular methods. The universal standard keys are used for

morphological identification of nematodes. Morphological identification needs careful processing, fixing and mounting nematodes. The extracted nematodes are concentrated in small quantity of water and killed by pouring hot water (65° to 85°C) over them and then fixed in 4% formaldehyde solution. For taxonomic studies, nematode are dehydrated by adopting the method of Seinhorst (1959) and subsequently mounted on glass slides in anhydrous glycerine for light microscopic studies. Nematodes are later observed under microscope, measurements are taken using an ocular micrometer and de Man's formula is used to determine pertinent values of measurements.



***Aphelenchoides besseyi*: A-** nematode suspension; B- anterior region; C- posterior region; D- tail terminus bearing **mucro** with pointed processes

Morphological identification is now days supplemented with bio-chemical and molecular methods. Biochemical approaches include the separation of proteins, enzyme analysis, esterase profiling etc. Molecular methods are more useful for detection, characterization and study of inter- and intra-specific variability in nematodes. The techniques such as the polymerase chain reaction, restriction fragment length polymorphism, randomly amplified polymorphic DNA, amplified fragment length polymorphism satellite-DNA probes, sequence-characterized-amplified regions (SCAR), high-resolution-melting-curve analysis, real-time-PCR assays and DNA sequencing are being used for nematode characterization and identification.

SECTION IV: Detection and Identification of Insects and Mites

Kavita Gupta, SP Singh, T Boopathi and DS Meena

The various techniques used for detection of insect and mite pests are as follows:

Detection of Infestation

Visual examination- Seeds / planting materials were examined by naked eye or with the help of magnifiers. The external infestation was detected by presence of external symptoms of insect damage i.e. holes, rotting, swelling or any other deformity etc or presence of dead or live insects/mites, eggs/egg shells, immature stages, exuviae or excreta thereof.

Visual symptoms of insect damage are as follows:

- Seeds- external and hidden infestation
- Skeletonization, cutting, wilting and curling up of the leaves
- Scarification of leaves
- Tunneling girdling of underground plant parts
- Tunneling/boring of stem, root, fruit /pod etc.
- Damage to flowers and consequent reduction in seed production
- Destruction, distortion, blotching, necrosis and proliferation of plant tissues
- Gall formation- galls, bud galls, twig galls
- Reduction in the leaf assimilative area
- Premature fruit fall
- Big bud
- Vagrant witches broom

Important insect orders from quarantine viewpoint are: 1. Coleoptera, 2. Lepidoptera, 3. Hymenoptera, 4. Homoptera, 5. Heteroptera, 6. Orthoptera, 7. Diptera and 8. Acarina.

X-ray radiography- Seed samples of the listed plant genera known to carry hidden infestation of bruchids, chalcids and others were subjected to X-ray radiography. Seeds were arranged on paper, kept over a black envelope loaded with an unexposed X-ray plate and exposed to soft X-rays, at 22 Kv, 3 mA for 15 seconds at the distance of 30 cm. The X ray film is then developed in a dark room to reveal the insect infestation. Meanwhile the paper on which seeds are arranged is kept undisturbed and the developed X-ray film is compared with the seed geometry, the infested seeds are handpicked.

Composition of the solutions used in developing and fixing the image on X ray film is as follows:

Chemicals for preparing developer (for 1 litre) for the X-ray films

- Metol Agfa =3.5gm
- Sodium sulphate anhydrous=60.0 gm
- Hydroquinone= 9.0 gm
- Sodium carbonate= 40.0 gm
- Potassium bromide= 3.5gm
- Distilled water to make volume 1 litre

(Note: Dissolve in an approximately 750 ml of water and make upto 1000ml.)

Chemicals for fixing the developed X-ray films (1 litre)

- Sodium thiosulphate-250gm
- Potassium metabisulphite-15 gm
- Distilled water to make volume 1 litre

(***Note:*** Dissolve in an approximately 750 ml of water and make upto 1000ml.)

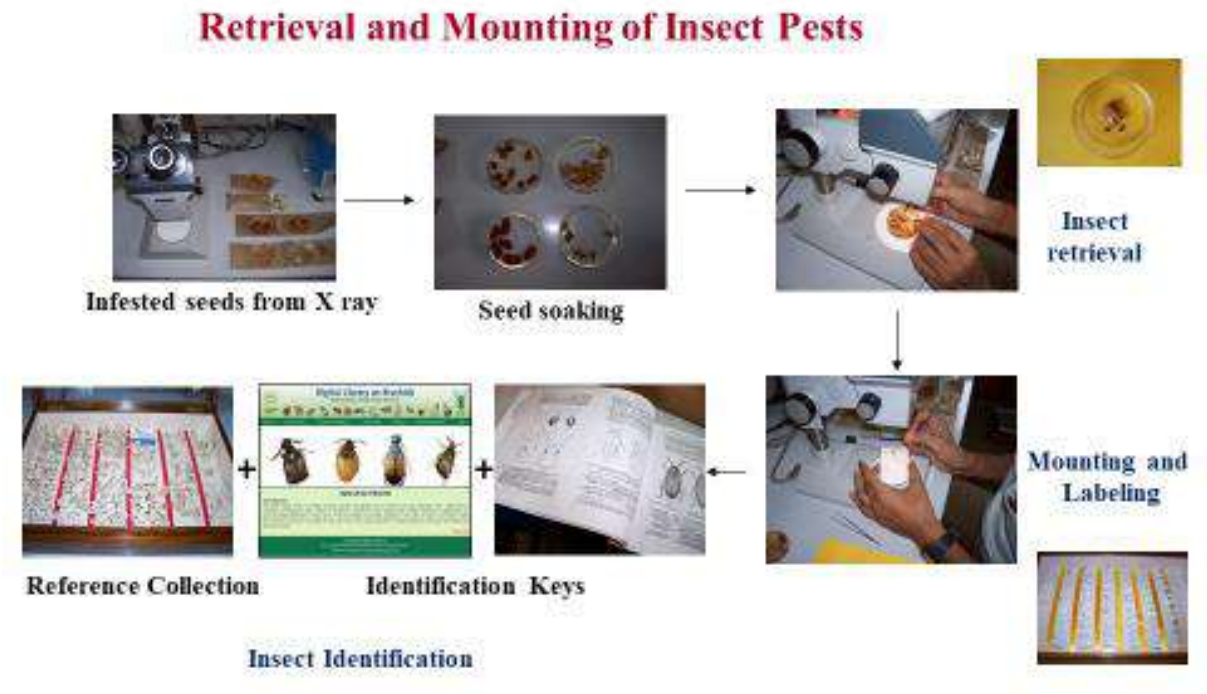
Of late a real-time X-ray system is being used wherein, the X-ray can be visualized directly on the computer monitor and picking of infested seeds taken up

Transparency technique- Small seeds like grasses which are difficult to salvage through X-ray radiography, are sampled and the representative sample is rendered transparent by heating in lactophenol-acid fuchsin (phenol 2 parts, lactic acid 2 parts, glycerin 1 part, distilled water (hot) 2 parts, little acid fuchsin) for detection of internal infestation (Kaura, 1959). The transparent seeds can easily be detected and appropriate salvaging method is adopted.

Mounting of mites- Slide mounts are prepared for the mites intercepted and they are then identified using identification keys. Some of the mites are semi transparent or transleucent, which are stained prior to mounting for easier identification.

Identification of Interceptions

The infested seeds separated by handpicking after X-ray radiography are soaked overnight, the seed teased open and the insect removed from inside. Based on the morphological and key characters the pests are identified using an identification key.



SECTION V: Detection and Identification of Weeds

Mool Chand Singh

Inspection and identification of weeds

In order to prevent the introduction of serious exotic weeds or of biotypes of existing weeds with their material, all imported plant/plant materials are subjected to critical examinations and only healthy material is released. Samples are examined for weed seeds by sieving through sieves of different pore sizes. Then they are critically and thoroughly examined using available standard methods such as-

Visual examination

Working samples are spread in a thin uniform layer on a clean white drawing sheet or in a white enamel tray and examined with the help of illuminated magnifier and all weed seeds are collected. Weed seeds are segregated into different types on the basis of their shape, size, colour, texture and the presence of any attachment. Segregated weed seeds are kept in screw caped vial and reference is written on the vial.

Microscopic examination

If the seeds are extremely small in size then stereoscopic binocular is used for weed seeds detection the seeds are placed in a glass *Petri* dish and examined under stereoscopic binocular

microscope and weed seeds are segregated from the crop seeds with the help of forceps or camel hair brush.

Identification of weed seeds

Seed identification is a specialised field of taxonomy that has been developed over the past 50 years to meet the problem of labelling the crop and weed seed. Accurate identification of weed seeds is necessary for the correct labelling of weed seed and also for correct quarantine action. This requires skill and good judgement on part of seed analyst making the examination .The seeds of some kinds of plants are sufficiently distinctive that they are not easily confused with these of other kinds and their identification posses no problems .There are many groups of plants, however, in which seeds of one species may closely resemble those of another species. In some cases one of these may be a crop plant and the other an undesirable or noxious weed like mustard and *Argemone mexicana* seeds.

Aids for identification

- With naked eyes, if seeds are large enough.
- Hand lens, if seeds are not clearly visible with naked eyes.
- Binocular microscope for extremely small seeds.
- Identification manual with colour photographs, drawings & description of important characteristics.

Identification techniques

Identification on the basis of morphological character

A collection of identified weed seeds is maintained as reference. The intercepted weed seeds are compared with reference collection for identification. Weed seeds are also identified on the basis of morphological characters by consulting identification keys.

Clues for identification

Following morphological characters of the seeds are more useful clues for identification.

- Shape
- Size
- Peculiarities of surface structures like smooth or rough, pitting grooves, sculpturing.
- Colouring characteristics of hilum (attachment scar) , mainly its shape, size and position
- Other noticeable characters like wings/ pappus , spines, awns, hairs.

- Sometimes similar external morphological characteristics of different families or genera make identification difficult. hence ,internal character are examined for proper identification .These internal clues include:-
- Endosperm: amount, position and shape.
- Absence of endosperm.
- Embryo: size, shape, cotyledon, development, position, textures, thickness and inner markings of seed wall.

Identification on the basis of vegetative and floral characters

If the weed seeds could not be identified on the basis of morphological characters, such seeds are grown in net houses in isolation under strict plant quarantine conditions to observe various vegetative and floral characters and on the basis of these characters, the weed species are identified.

Weed species of Quarantine significance to India

Government of India has strengthened the existing system and brought into force, the new Plant Quarantine (Regulation of Import into India) Order 2003. Enforcement of this order is mainly intended to prevent the introduction and spread of exotic pests that are destructive to the country. According to the special provisions for Quarantine weeds (clause 3(12) and (Schedule VIII) of Plant Quarantine (Regulation of Import into India) Order 2003, no consignment of seed or grain contaminated with Quarantine weeds shall be permitted unless devitalized. Thirty-two weed species, which are listed in Schedule VIII are *Allium vineale, Ambrosia maritima, Ambrosia psilostachya, Ambrosia trifida, Apera-spica-venti, Bromus secalinus, Cenchrus tribuloides, Centaurea diffusa, Centaurea maculosa, Centaurea solstitialis, Cichorium pumilum, Cichorium spinosum, Cordia curassavica, Cuscuta australis, Cynoglossum officinale, Echinochloa crus-pavonis, Froelichia floridana, Helianthus californicus, Helianthus ciliaris, Heliotropium amplexicaule, Leersia japonica, Matricaria perforatum, Polygonum cuspidatum, Proboscidea lovisianica, Salsola vermiculata, Senecio jacobaea, Solanum carolinense, Striga hermonthica, Thesium australe, Thesium humiale* and *Viola arvensis.*

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20

Conservation Procedures for Seed Genebank

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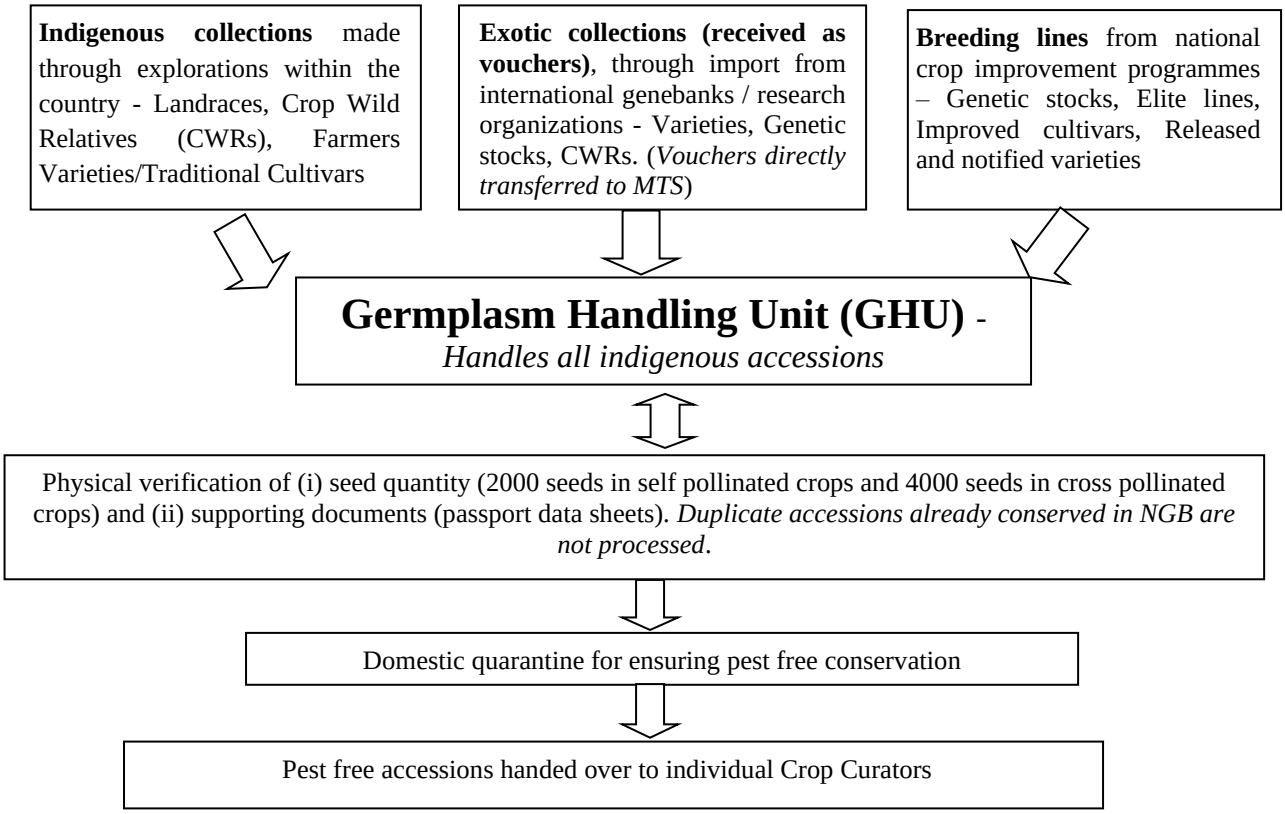
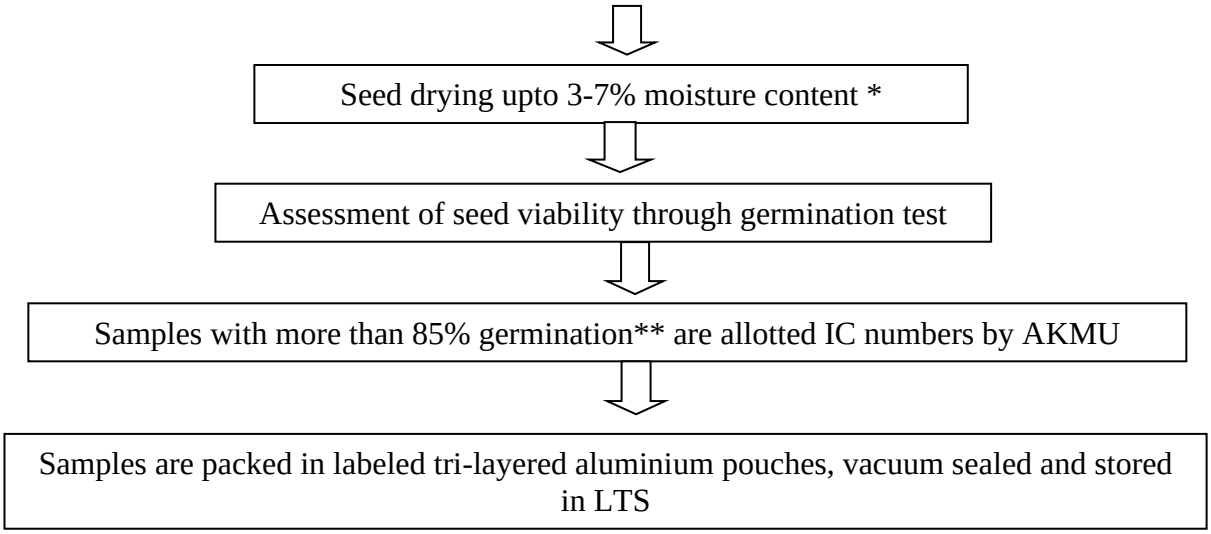
The *Ex-situ* form of conservation involves various strategies, based on the seed storage behaviour and mode of reproduction of the species. Seed conservation is the most cost-effective and practically feasible procedure amongst all the conservation techniques Hence, all species producing orthodox seeds, which can be safely dried to low moisture content without loss of viability and can survive sub-zero temperatures, are conserved in the form of seeds in all genebanks. The National Genebank located at ICAR-NBPGR has more than 4.4 lakh accessions, belonging to 1945 species, conserved in the form of orthodox seeds. This chapter describes the various procedures and protocols involved in the processing of these seed germplasm accessions for long term conservation.

Flow Chart for Seed Germplasm Conservation

Acquisition and Processing of Germplasm in National Genebank





*Under the standard drying procedure in NGB, done at 15% RH and 15ºC, oil seeds equilibrate to approx. 3% and starchy seeds equilibrate to approx. 7%
**In vegetables/Medicinal/Forage/Rare endangered species, relaxed upto 50-70%

S.No.	Descriptor	Definition
1	Wild	All species other than cultivated of PGR values/ potential value.
2	Landrace/Traditional cultivar	All cultigens in farmer's field with/without species names frequently associated with unique traits and geographical region identified by farmers.
3	Breeding/Research material	Semi-finished products or segregating material generated out of breeding program to meet specific objectives.
4	Genetic stock	Trait and gene specific germplasm, experimentally developed or identified through scientific interventions (e.g. sources of resistance, cytogenetic stock, mutants, mapping population, MS lines.
5	Elite line	Crop varieties under advanced varietal trait but not yet released/ notified. Selection from population from the coordinated trail (AVT I & II line).
6	Improved cultivar	Improved cultigens of common knowledge that are in commercial cultivation (extant varieties and varieties released by institution/ organization/ State.
7	Released variety	Varieties released and notified by the central Sub-committee on Crop Standards, Notification and Released of Varieties of Agricultural and Horticultural Crops.

I. Drying of germplasm

For long term conservation, seeds of each accession should be dried to a moisture content of 3-7%, depending on the species. At this moisture level, the seed tissues will contain only the tightly bound water molecules which do not contribute significantly to the ageing

metabolism. For attaining the recommended moisture level, the seeds are dried at 15°C temperature and 15% relative humidity in standard walk-in or batch dryers. The duration of drying will vary with the species. Oil seeds dry rapidly and attain 3% moisture content within 7 days, whereas maize seeds require more than 20 days to dry upto 7% moisture content.

Seed Moisture Testing

After the anticipated drying duration, the samples are subjected to moisture testing using the crop-specific procedure, as recommended by the International Seed Testing Association (ISTA). As per ISTA rules, the only acceptable method for seed moisture testing is the Constant Temperature Oven Drying method. However, for preliminary screening, curators can use a well calibrated digital moisture meter. For the final moisture determination, ISTA protocol involving oven drying should be strictly adhered to.

Pre-drying

If in the initial screening, the seed samples are suspected to contain above 17% (10% for soya bean and 13% for rice), a pre-drying should be conducted before going ahead with the oven-drying.

If pre-drying is required, the following procedure should be adopted:

1. Weigh approximately 4–5 g of seeds in two replicates (taken in containers of known weight).
2. Pre-dry the samples by leaving it open overnight in a warm, dry place.
3. Weigh them again (along with their containers) and determine the loss of weight (loss of moisture) by subtraction.
4. Calculate the moisture content on a fresh-weight basis.

The percentage of moisture content on weight basis may be calculated by using the formula as mentioned in the Work sheet

After completing pre-drying, further moisture testing should be done for these samples using Oven-drying method. If moisture content of the received sample is apparently below 17%, the accessions may be directly subjected to oven drying.

Materials required for oven drying

a) Grinding mill
b) Constant temperature oven
c) Moisture bottles made of glass or non-corrosive metal
d) Desiccator and silica gel
e) Analytical balance
f) Seed material

Procedure

The constant temperature oven method is the standard protocol for seed moisture testing in genebanks. Prior to drying, grinding is obligatory for large seeds, seeds with seed coats that impede water loss from the seeds and oily seeds (Table 1). The samples are always tested in two replicates. There are two methods of oven drying-

Low constant temperature oven method: This method is generally used for moisture estimation of oily seeds (Table 2). The seeds in moisture bottles with their lid open are placed in an oven maintaining a temperature of 103 ± 2^0C for 17±1 hours. The lids and bottles should be properly labeled. After the recommended duration, replace the lids within the oven itself and shift the bottles to a desiccator containing silica gel, for cooling. The seeds in the bottles are then weighed, along with the lid.

High constant temperature oven method: This method is generally recommended for the seeds of field crops (Table 3). The procedure is same as above except that the seeds are placed in the oven at 130±20C for 2-4 hours.

Calculation of results: Calculation of seed moisture content is done using Eqn 1. All the weights should be taken in grams upto three decimal places and the result should be reported to the nearest 0.1%.

If the seeds required pre-drying, the moisture content is calculated twice i.e once after pre-drying and secondly, using oven method. If S1 is the percentage moisture lost in the pre-drying, and S2 is the percentage moisture lost in the second stage, then the original moisture content of the sample calculated as a percentage is:

$$(S1 + S2) - \frac{S1 \times S2}{100}$$

Table 1: Species for which grinding is obligatory

Arachis hypogaea	*Oryza sativa*
Avena spp.	*Phaseolus* spp.
Cicer arietinum	*Pisum sativum*
Citrullus lanatus	*Quercus* spp.
Fagopyrum esculentum	*Ricinus communis*
Fagus spp.	*Secale cereale*
Glycine max	*Sorghum* spp.
Gossypium spp.	*Triticum* spp.
Hordeum vulgare	*Vicia* spp.
Lathyrus spp.	*Zea mays*
Lupinus spp.	

Table 2: Species for which the low constant temperature oven method shall be used

Allium spp.	*Raphanus sativus*
Arachis hypogaea	*Ricinus communis*
Brassica spp.	*Sesamum indicum*
Camelina sativa	*Sesamum orientale*
Capsicum spp.	*Sinapsis* spp.
Glycine max	*Solanum melongena*
Gossypium spp.	
Linum usitatissimum	

Table 3. Species for which the high constant temperature oven method shall be used

Agrostis spp.	*Lactuca sativa*
Alopecurus pratensis	*Lathyrus* spp.
Anethum graveolens	*Lotus* spp.
Anthoxanhum odoratum	*Lupinus* spp.
Anthriscus spp.	*Lycopersicon lycopersicum*
Apium graveolens	*Medicago spp.*
Arrhenatherum spp.	*Nicotiana tabacum*
Asparagus officinalis	*Oryza sativa*
Avena spp.	*Panicum* spp.
Beta vulgaris	*Papaver somniferum*
Bromus spp.	*Phalaris* spp.
Cannabis sativa	*Carum carvi*
Phaseolus spp.	
Chloris gayana	*Pisum sativum*
Cicer arietinum	*Poa* spp.
Secale cereale	
Citrullus lanatus (C. vulgaris)	*Sorghum* spp.
Cucuburbita spp.	*Trifolium* spp.
Cuminium cyminium	*Triticum* spp.
Cynodon dactylon	*Vicia* spp.
Cynosurus cristatus	*Zea mays*
Dactylis glomerate	*Daucus carota*
Fagopyrum esculentum	*Hordeum vulgare*

When the seeds are found to have attained moisture content of less than 7%, the accessions are tested for their seed viability.

Recording and Calculation of Seed Moisture Content

Accession no.	Replicate/ Moisture Bottle no.	Wt of empty Moisture Bottle with lid (g)	Wt of Moisture Bottle with lid + seed before drying (g)	Wt of Moisture Bottle with lid + seed after drying (g)	Moisture content % (wb)	
		W1	W2	W3	(W2-W3)/ (W2-W1) × 100	Average (R I + R II)/2
	R I					
	R II					
	R I					
	R II					
	R I					
	R II					
	R I					
	R II					

Example:

Calculation:

Rep 1: % Moisture content = (**W2-W3)/(W2-W1) × 100**

Rep 2: Moisture content =(**W2-W3)/(W2-W1) × 100**

Moisture content (fresh-weight basis) = (**%MC Rep.1+ %MC Rep.2)/2**

II. Testing for Seed Viability

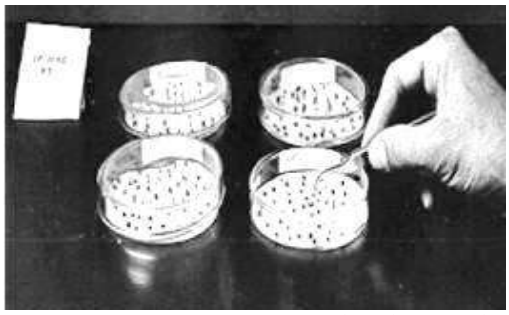
Seed viability is assessed through the standard germination test, which is the easiest and most reproducible mode of viability testing. For long term conservation in seed gene banks, it is mandatory that the initial seed viability of conserved samples should not be less than 85%. Exceptions may be granted for some specific accessions of horticultural crops, forestry species, forage grasses and crop wild relatives, due to their unique reproductive biology and growth cycle. Wherever the exemptions from genebank standards are granted, the Indian Minimum Seed Certification Standards (IMSCS) is followed.

Standard Germination Test

The germination test is conducted as per the rules and procedures prescribed by ISTA. However, a compromise in seed quantity is permitted since availability of seed in submitted samples of germplasm is significantly lesser than commercial samples received in seed testing laboratories. Twenty five seeds in two replications are used in exceptional cases where seed quantity is extremely low. Two basic methods are used for testing the germination of germplasm seeds.

***Top of Paper Method*:** This method is adopted for small seeded crops such as mustard, amaranth, etc. Two layers of filter papers, moistened with distilled water, are placed in petri plates that are labeled with accession number, replication number and date of plating. Seeds are plated on the paper and the petri plates are incubated in germinators maintained at high humidity and appropriate /optimum temperature as recommended by ISTA (Table 1). Germination evaluation is done according to ISTA rules (ISTA, 2019).

Between Paper Method: This method is suited for larger seeded crops such as green gram, chickpea, *etc.* Seeds are germinated between two layers of moist crepe papers, by arranging them in rows at regular intervals and leaving sufficient gap on both sides. It is then over-layered with a second layer of moistened crepe paper, followed by a wax paper. The required labelling is done on the wax paper and they are rolled loosely, with the wax paper on the outer side. The paper rolls are placed in trays containing distilled water, to keep the papers moist during the test duration. The tray is placed in germinator maintained at recommended temperature.




Germination testing (Top of paper method)


Germination testing (Between paper method)

Sand is also used as germination medium in case of large sized seeds and also when fungal infection or phytotoxicity of paper medium is anticipated

Seedling Evaluation

Germination evaluation is done according to ISTA rules. Seedlings are assessed on the day designated for final count and are classified as normal seedlings, abnormal seedlings, fresh ungerminated seeds and dead seeds.

Abnormal seedlings

Seedlings with the following defects are classified as abnormal-

Damaged: seedlings with any of the essential structures missing or so badly and irreparably damaged that balanced development cannot be expected. For *eg.*, missing, broken, or split primary root, Shoot that is split right through or missing, separated or missing cotyledons. Absence of seminal roots in the case of monocots is also considered abnormal.

Deformed or unbalanced: seedlings with weak development or physiological disturbances or in which essential structures are deformed or out of proportion. For *eg.*, stubby, stunted, split or glassy roots and shoots, necrotic cotyledons and deformed terminal bud/leaves.

Decayed*:* seedlings with any of their essential structures so diseased or decayed as a result of primary infection that normal development is prevented.

Seedlings which are decayed by fungi or bacteria are classified as normal, if it is evident that the parent seed is not the source of infection, and if it can be determined that all the essential structures were present.

A) Seedling evaluation B) Normal and abnormal seedlings

Fresh Ungerminated seeds

When 5% or more of fresh ungerminated seeds are present, their viability should be determined through quick viability testing, as descibed in section III. A high percentage of

fresh ungerminated seed is an indication of dormancy being prevalent in the sample. ISTA has recommended specific dormancy breaking treatments for each species, based on the nature of dormancy. The most common type of dormancy in agri-horticultural crops is the physiological dormancy. Physical dormancy is also prevalent in certain families of cultivated crops like fabaceae, malvaceae and anacardiaceae. The dormancy breaking treatments recommended by ISTA for major crop species is mentioned in Table 4. After exposing the seeds to the relevant treatment, the germination test should be repeatedusing standard procedure, for noting the final viability percentage.

Reporting of results

The result of the germination test is expressed as percentages by number of normal and abnormal seedlings and hard, fresh and dead seeds. The percentages are rounded to the nearest whole number. The final viability percentage is the average percentage of normal seedlings in the sample.

Table 4: Germination methods for some agricultural and horticultural seeds

Species	Substrata	Temp °C	Final Count	Recommendation for breaking dormancy
Abelmoschus esculentus(Okra)	TP;BP	20-30	21	--
Arachis hypogaea (Groundnut)	BP;S	20-30;25	10	Remove shells,
Allium cepa (Onion)	BP;TP	20;15	12	Prechill
Avena sativa (Oat)	BP;S	20	10	Preheat (30-35°C; prechill, GA_3)
Beta vulgaris (Sugar beet)	TP;BP;S	20-30; 15-25	14	Prewash 2-4 hours in running water
Brassica juncea (Sarson)	TP	20-30;20	7	Prechill; KNO_3
Brassica juncea (Rape seed)	TP	20-30;20	7	Prechill
Brassica oleracea (Cabbage, Cauliflower)	TP	20-30;20	10	Prechill, KNO_3
Cajanus cajan (Red gram)	BP;S	20-30;25	10	--
Capsicum sp. (Chilli)	TP;BP	20-30	14	KNO_3
Cicer arietinum (Bengal gram)	BP;S	20-30;20	8	--
Corchorus sp .(Jute)	TP;BP	30	5	--
Cucumismelo (Muskmelon)	BP;S	20-30;25	8	Low moisture
Cucumissativus (Cucumber)	TP;BP;S	20-30;25	8	--
Cucurbita moschata (Pumpkin)	BP;S	20-30;25	8	--
Cucurbita pepo (Summer squash)	TP;BP;S	20-30;20	14	--
Daucuscarota (Carrot)	TP;BP;S	20-30;20	14	--
Glycine max (Soybean)	BP;S	20-30;25	8	--

Gossypium Sp.(Cotton)	BP;S	20-30;25	12	--
Helianthus annus (Sunflower)	BP;S	20-30;25; 20	10	Preheat, prechill
Hordeum vulgare (Barley)	BP;S	20	7	Preheat (30-35°C) prechill, GA_3, KNO_3
Lactuca sativa (Lettuce)	TP;BP	20	7	Prechill
Lens culinaris (Lentil)	BP;S	20	10	Prechill
Linum usitatissimum (Linseed)	TP;BP	20-30;20	7	Prechill
Lycopersicon lycopersicum (Tomato)	TP;BP	20-30	14	KNO_3
Nicotiana tabacum (Tobacco)	TP	20-30	16	KNO_3
Oryza sativa (Paddy)	TP;BP;S	20-30;25	14	Preheat (50°C), soak in H_2O or HNO_3 24 hr
Pennisetum typhoides (Pearlmillet)	TP;BP;	20-30	7	--
Pisum sativum (Pea)	BP;S	20	8	--
Ricinus communis (Castor)	BP;S	20-30	14	--
Sesamum indicum (Sesame)	TP	20-30		--
Solanum melongena (Brinjal)	TP;BP	20-30	14	--
Sorghum vulgare (Jowar)	TP;BP	20-30	14	--
Triticum aestivum (Wheat)	TP;BP;S	20	8	Preheat (30-35°C) prechill, GA_3
Vicia faba (Broad bean)	BP;S	20	14	Prechill
Vigna mung o(Black gram)	BP;S	20-30;25; 20	7	--
Vigna radiata (Green gram)	BP;S	20-30;25	7	--
Vigna unguiculata(Cowpea)	BP;S	20-30;25	8	--
Zea mays (Maize)	BP;S	20-30;25; 20	7	--

TP: Top of Paper; BP: Between paperm, S: Sand, TS: Top of Sand, GA_3: Gibberellic acid (0.05 to 0.1%)
KNO_3: Potassium nitrate (0.2%), HNO_3: Nitric acid (1 N)

DATA SHEET FOR RECORDING GERMINATION RESULTS

Crop/species: **Temperature:**

Accession number: **Light:**

Date of storage: **Special treatments**

Date of testing: **Incubation time:**

Substrate:

Replication	Days	Normal seedlings				Total	Remarks
		I	II	III	IV		
No. of seeds tested							
Date							
	1						
	2						
	3						
	4						
	5						
	6						
	7						
Abnormal							
Hard/Fresh Ungerminated							
Dead							
Speed of Germination							

Data sheet for recording Seed Vigour results

Accession No.		Germination %	Seedling Length										Mean Seedling Length (S1+S2+S3+S4+S5+S6+S7+S8+S9+S10) /10	Seedling Dry Weight (10 Seedlings) g	Vigour Index (% Germination XSeedling Length)
			S1	S2	S3	S4	S5	S6	S7	S8	S9	S10			
	R1														
	R2														
	R3														
	R4														

Replication	Days	Normal seedlings				Total	Remarks
		I	II	III	IV		
No. of seeds tested							
Date							
	1						
	2						
	3						
	4						
	5						
	6						
	7						
Speed of Germination							

Data sheet for recording Electrical Conductivity

Accession No.		Number of seeds	Weight of Seeds	EC of Distilled Water	EC of Seed leacheate - Reading of distilled water	EC of Seed leacheate - Reading of distilled Water mu *mhos/cm/g*
	R1					
	R2					
	R3					
	R4					

III. Quick Viability Testing

Tetrazolium (TZ) assay is the fast evaluation for seed viability and alternative quick method for seed germinability. All respiring tissues are capable of converting a colourless compound, TZ (2,3,5 triphenyl tetrazolium chloride) a carmine red coloured water-insoluble formazan by hydrogen transfer reaction catalysed by the cellular dehydrogenases. TZ enters both living and dead cells but only living cells catalyse the formation of formazan which being non-diffusible. It stains the viable tissue red whereas the absence of respiration prevents formazan production making the dead tissue remain unstained.

Materials and Reagents:

- Seeds
- 2,3,5 triphenyl tetrazolium chloride
- Phosphate buffer (pH 7)
- Distilled water
- Clearing agent (lactophenol solution)
- Glycerine for Mounting
- Incubator

- Weighing balance
- pH meter
- Stereo Microscope

Procedure:

- Add 1 g 2,3,5 triphenyl tetrazolium chloride in 100 ml distilled water in amber colour bottle. Mix well and store in dark at 4 °C (can be kept for several months under such conditions).
- The pH of the TZ staining solution should be 7. Solution with pH > 8 or pH < 4 would result in either intense staining or would not stain even viable seed tissues. If water is out of neutral range then use phosphate buffer with pH 7 to dissolve TZ.
- Seeds to be tested are soaked in water overnight. Once imbibed, they're cut in half with a scalpel to expose the embryo. The seed coats of larger seeds (like legume seeds) should be removed before examination. The incubation time varies with seed type and morphology and needs to be standarized for individual species. Temperatures between 20-40°C have no adverse effect on tetrazolium test accuracy. Staining rate increases as temperature increases. Tests can be performed at room temperature, but will take longer to stain.Temperatures greater than 40° C should not be used. The general rule of thumb is that staining will take place twice as fast at 30° C than at 25° C, and twice as fast at 35° C than at 30° C.
- After staining, wash the seeds 2-3 times with distilled water.
- Immerse the stained seeds in clearing agent for 1-2 h. if the pigment within the seed coat prevents clear vision after staining
- Observe the seeds under stereo microscope.
- Evaluate the seeds on the basis of staining pattern and colour intensity. Among stained seeds, seeds with bright red staining are completely viable while partially stained seeds may produce either normal or abnormal seedlings. Pink or greyish red stain indicates dead tissue. Completely unstained seeds are non-viable.
- When performed appropriately, the percentage of viable seeds obtained by tetrazolium assay is very close to the percentage of seed germination expected under most favourable conditions.

Different pattern of TZ staining showing visible, abnormal and dead or non-viable seeds. TZ staining pattern on *Jatropha curcas*

(1). Seeds completely and uniformly stained (2) seeds showing minor unstained areas on cotyledon opposite to deeply stained radicle tip and parts of cotyledon (3) Seeds showing necrotic tissue towards the radicle tip and in the centre of the cotyledon (4) seeds completely unstained.

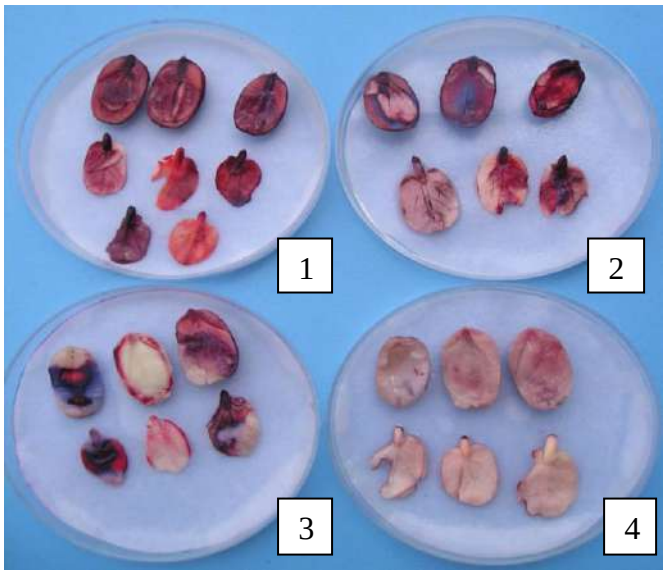


Table 5: Concentration, temperature and duration of staining with tetrazolium solution in various crops*

Crop	Species	Preconditioning	Staining
Barley	*Hordeum vulgare*	Imbibe or soak, 6–18h	0.5%, 3h, 30°C
Beans	*Phaseolus* spp.	Imbibe 18–24h, then soak, 2–3h	0.5–1%, 6–24h, 30°C
Brassica	*Brassica* spp.	Imbibe or soak, 16–18h	0.5–1%, 3–6h, 30°C
Chickpea	*Cicer arietinum*	Imbibe or soak, 18h	1%, 6–24h, 30°C
Cowpea	*Vigna unguiculata*	Soak, 22h	0.5–1%, 16–24h, 30°C
Eggplant	*Solanum melongena*	Imbibe or soak, 18h	0.5–1%, 6–24h, 30°C
Faba bean	*Vicia faba*	Soak, 22h	0.5–1%, 16–24h, 30°C
Finger millet	*Eleusine corocana*	Soak, 18h, 5°C	0.5%, 3h, 30°C
Lentil	*Lens culinaris*	Imbibe, 18h, then soak, 2–3h	1%, 6–24h, 30°C
Maize	*Zea mays*	Imbibe or soak, 18h	0.5–1%, 2–6h, 30°C
Pea	*Pisum sativum*	Imbibe 18–24h, then soak, 2–3h	0.5–1%, 6–24h, 30°C
Pearl millet	*Pennisetum glaucum*	Imbibe or soak, 6–18h	0.5–1%, 6–24h, 30°C
Rice	*Oryza sativa*	Imbibe or soak, 18h	0.5%, 3h, 30°C
Rye	*Secale cereale*	Imbibe or soak, 6–18h	0.5%, 2–3h, 30°C
Sorghum	*Sorghum bicolor*	Imbibe, 16h, 30°C	0.5–1%, 0.5–1h, 40°C
Sugar beet	*Beta vulgaris*	Imbibe or soak, 16–18h	1%, 24–48h, 30°C
Sunflower	*Helianthus annuus*	Imbibe or soak, 18h	0.5–1%, 3–6, 30°C
Triticale	*Triticosecale*	Imbibe or soak, 6–18h	0.5%, 2–4h, 30°C
Wheat	*Triticum aestivum*	Imbibe or soak, 6–18h	0.5%, 2–4h, 30°C

*** Sorce: *Rao et.al. (*2006) Manual of Seed Handling in Genebanks**

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21

Strategies for *in vitro* Conservation and Cryopreservation of Germplasm

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In vitro culture techniques have been used in plants since decades. *In vitro* conservation refers to maintenance of germplasm in culture vessels in artificial media, under sterile conditions in controlled environments. Aseptic establishment, multiplication and regeneration of plant parts (explant) into complete plants are prerequisite of successful *in vitro* conservation. These techniques are particularly applied to vegetatively propagated crops, crops which are sterile or crops whose seeds cannot be conserved through standard seed storage methods. Conservation of germplasm can be done either under slow growth conditions for medium-term storage in an *in vitro* active genebank (IVAG) or through cryopreservation for long-term storage in *in vitro* base genebank (IVBG). The following strategies are mainly used for *in vitro* conservation:

Slow Growth

In this strategy, the growth rate of *in vitro* cultures is reduced to extend subculture duration. Subculture duration can be extended up to 1-2 years, thus reducing the time, labor, and materials required to maintain the cultures. This can be achieved by reducing the temperature/ light, including growth retardants or osmotica in culture medium, modifying media/ gaseous environment, induction of storage organs etc.

Three basic steps involved in this strategy are; i) aseptic culture establishment, ii) shoot multiplication and iii) *in vitro* conservation.

Aseptic culture establishment

Following steps are followed for aseptic culture initiation and establishment:

1. Prepare suitable culture medium (MS medium, White's medium, B_5 medium) containing inorganic and organic nutrients, growth hormones and gelling agent. Inorganic nutrients are classified in two groups based on their required concentrations, namely, macronutrients (six elements, namely, nitrogen, phosphorus, sulphur, calcium, potassium and magnesium) and six micronutrients including iron, molybdenum, copper, zinc, boron and manganese. Organic nutrients include carbon

source (sucrose, fructose and other carbohydrates) and nitrogen source (vitamins and amino acids). Thiamine is an essential vitamin whereas inositol, pyridoxin, nicotinic acid, pantothenic acid etc. are also important. Auxins, cytokinins and Gibberellins are the most commonly used growth hormones. Agar is most commonly used gelling agent used for solidification of culture medium. All the necessary components are incorporated in culture medium and then sterilized.

2. Select suitable explants: Any plant part like stem segments, nodal sections, axillary buds, shoot/ root tip, leaf, embryo, anther, and ovary, may serve as explant. Explant is always taken from young and healthy plant parts.
3. Sterilize the explant by a process known as surface sterilization: explants are sterilized by using different types of disinfectants like sodium hypochlorite (2%), silver nitrate (1%) calcium hypochlorite (9-10%), bromine water (1-2%), mercuric chloride (0.1-1%), hydrogen peroxide etc. and different types of antibiotics. Due to toxicity of sterilizing agent towards plant cells, the concentration of sterilizing agent and duration of treatment is optimized to minimize cell death.
4. Inoculate the sterile explant on the surface of the solidified nutrient medium under aseptic conditions in laminar air flow.
5. Incubate at 25±2°C, with a relative humidity of 50-60% with 16 h of photoperiod.

Shoot multiplication

Once the aseptic cultures are established, protocol for shoot multiplication is standardized. For shoot multiplication, contamination-free cultures are sub-cultured every 4-6 weeks on shoot multiplication medium. For sub-culturing, the explant is dissected to required size using sterile surgical blade and forceps and implanted vertically onto the media. The cultures are maintained at 25±2°C, 16 h photoperiod and relative humidity of 50 to 60%.

In vitro conservation

In vitro conservation under slow growth conditions is done to minimize the growth of cultures thus increasing sub-culture intervals, without compromising its viability and genetic stability. Slow growth is usually achieved through modification of incubation conditions or using growth retardants/ osmotica in culture medium. For slow growth, explant is inoculated vertically onto thenormal or optimized slow growth medium (media containing osmotica, growth retardants *etc*). The cultures are maintained under optimized low temperature/ light conditions *e.g.* temperate crops can be stored at 4°C in dark and tropical crops can be stored at 15-20°C. The survival and regeneration ability of cultures is assessed periodically.

Cryopreservation

Cryopreservation (Greek, krayos-frost) literally means preservation in the frozen state. The principle involved in cryopreservation is to bring the plant cell or tissues to a metabolically

inactive and non-dividing state by freezing at ultra-low temperatures in the presence of cryo-protectants.Cryopreservation is mostly carried out using liquid nitrogen. At the temperature of liquid nitrogen (-196°C), the cells stay in a completely inactive state and thus can be conserved for long periods.

Basic steps in cryopreservation involves; i) development of sterile tissues, ii) addition of cryo-protectants and pre-treatment, iii) freezing, iv) storage, v) thawing, vi) re-culture, vii) measurement of survival/viability, viii) Plant regeneration

Formation of ice crystals inside the cells should be prevented during freezing as they cause injury to the organelles and the cell. This is usually achieved by either lowering the water content of cells by desiccation (air/ osmotic) or increasing cell viscosity by using cryoprotectants like dimethyl sulfoxide (DMSO). Commonly applied cryopreservation techniques are desiccation-freezing, encapsulation-dehydration, vitrification, encapsulation-vitrification and droplet-vitrification.

Desiccation-freezing

Desiccation-freezing is mainly employed for freezing seeds, embryos, dormant buds, pollen, undifferentiated cultures and shoot apices of cold-tolerant species. Whole seeds, dormant buds, pollen and embryos can be desiccated in the desiccator/ vacuum desiccator filled with charged silica gel. For Air-desiccation freezing, excised embryonic axes may be kept in batches of 20-25 in the sterile air flow of laminar flow cabinet immediately after excision. Desiccation freezing of dormant buds and pollen is described below:

Cryopreservation of dormant buds

Vegetative dormant buds in most of the fruit crops are utilized for propagation of identical clones since centuries and hence could be ideal for long term conservation of selected woody crops. This technique is being profusely utilized in case of conservation of fruit trees viz., *Malus* spp. (Forsline *et al.,* 1998; Sakai *et al.,* 1978), *Pyrus* (Suzuki *et al.,* 1997), *Morus* (Niino *et al.,* 1993) etc. Besides having the advantage of clonal integrity, this method is relatively simple, space efficient, amenable to long term conservation, costs effective when compared to new cryopreservation techniques. Bud sticks of desired length are first desiccated to the optimum moisture content at which its viability is intact and then cryopreserved followed by step wise freezing.

(a) Step wise freezing: Nodal sections, pre-desiccated to optimal moisture contents are kept in a refrigerator followed by sequentially lowering the temperature at -5°C/day till terminal temperature of -25°C (holding for 48 h) followed by a rapid immersion in LN.

(b) Thawing and rehydration of buds: This is the most critical step of recovery in dormant bud cryopreservation. The protocol of thawing must be standardized for each species. Basically two types of thawing are followed i.e., slow thawing and rapid thawing. In

slow thawing, the cryovials are simply removed from LN storage tank and placed in a refrigerator (4°C) for 24 hours before subjecting to rehydration, while in rapid thawing cryovials are suspended in lukewarm water (temperature at 37°C) for about 20-30 minutes followed by rehydration.

(c) Rehydration of buds: Buds/nodal sections must be rehydrated in moist peat moss for 10-15 days at 4°C temperature (in case of temperate species) or at room temperature (for tropical/subtropical species).

(d) Recovery of thawed buds: For *in vivo* recovery, chip budding can be done on a suitable rootstock in field and bud emergence and re-growth should be recorded. In case of test of *in vitro* recovery, bud pieces must be surface sterilized first followed by removal of 4-7 outer scales before culturing on a suitable nutrient media.

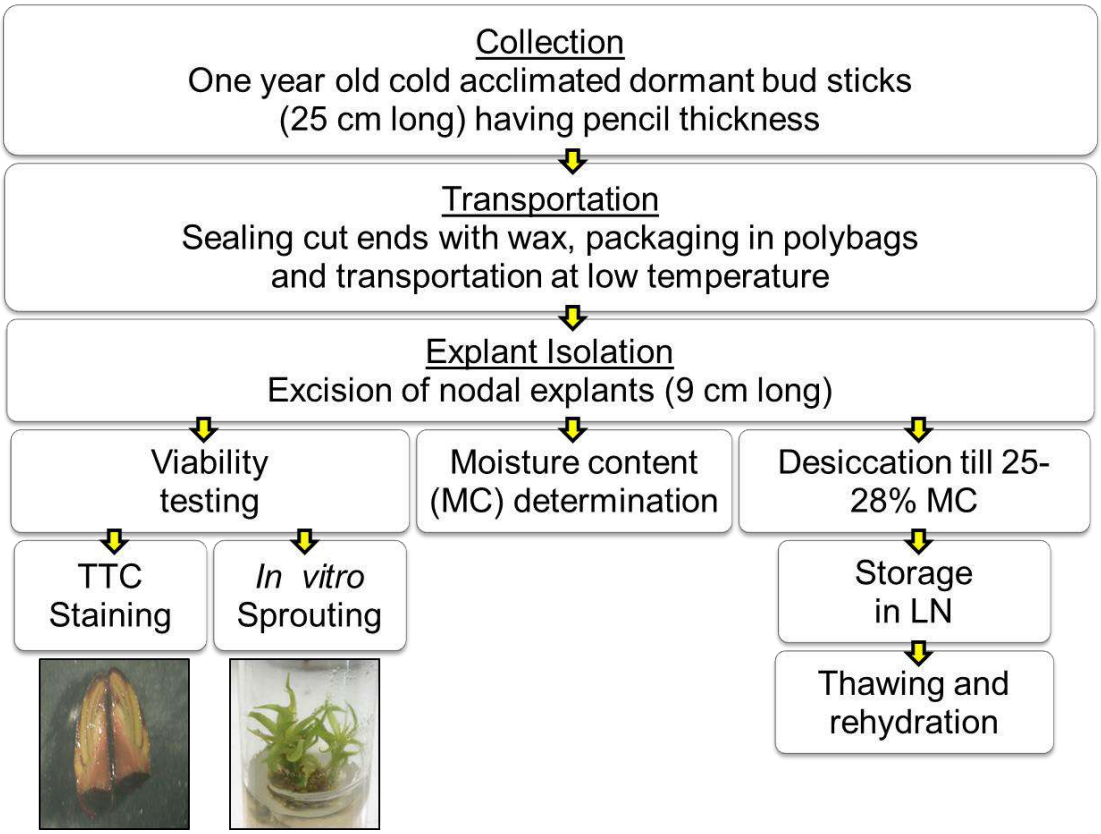

Figure 1: Basic steps in cryopreservation of dormant buds

Cryopreservation of pollen

Pollen cryopreservation is one of the complementary conservation strategies to seed or vegetative propagules and may be required for facilitating breeding programmes. Pollen grains are haploid male gametes that can be easily collected and stored in viable condition for a sufficiently long time for use throughout the year for breeding programmes and other studies (Shivanna and Rangaswamy, 1992; Chaudhury *et al*., 2010).

Pollen collection and extraction procedure

- Collection of the flowers should preferably be done in early morning or soon after anther dehiscence for optimal response.
- Anthers, if not dehisced properly should be placed on a butter paper sheet under a table lamp for few hours for collection of pollen.
- Sticky pollen can be extracted using organic solvents (usually cyclohexane) and passed through a filter paper via sterilized syringe (Chaudhary *et al.,* 2010). The filter paper containing pollen grains is then put in cryovials for storage in LN.

Viability tests

- In vitro germination is a simple method to study the physiology and biochemistry of pollen germination and pollen tube growth. Within a few hours pollen tubes grow *in vitro* and data are to be quantified.
- The composition of a germination medium to obtain optimal response has to be empirically formulated for each species. Apart from moisture, pollen generally require a carbohydrate source, boron and calcium for satisfactory germination and tube growth.
- A small amount of pollen is disbursed uniformly with a needle over a drop of pollen germination medium placed on a glass slide. Germination percentage is recorded after the prescribed duration of incubation (generally 3-6h) in a humid chamber, under the microscope. Pollen showing tube lengths longer than the pollen diameter are scored as viable with 400-500 pollen in two replicate drops scored in total.
- Fertilizing ability of cryostored pollen is observed in selected cases and fruit set is quantified. The best method to test the viability is by field pollination and quantifying it by seed set percentage.
- Pollen germinability after cryostorage is to be assessed using the same protocols used for fresh pollen. Images invariably are recorded using compound microscope.

Storage in liquid nitrogen (LN)

- Pollen desiccated to suitable moisture contents can be stored in cryovials, aluminum packets or gelatin capsules.
- Pollen in sufficient quantities can be stored in cryovials of 1 ml capacity and held at -196ºC (in liquid nitrogen) by placing into a canister of a cryotank. After 24 h, the cryovials may be shifted to the vapor phase of LN at temperatures between –170ºC to –180ºC.
- Samples may be removed from LN and thawed at room temperature for 30 minutes prior to viability testing.

Encapsulation-dehydration (ED)

The technique of cryopreservation by encapsulation-dehydration was developed in France by the team of Prof. Jean Dereuddre for pear and potato shoot-tips (Dereuddre *et al*., 1990; Fabre and Dereuddre, 1990). This method is based on the synthetic seed technology of encapsulating explants in calcium alginate beads. Once encapsulated, the explants are precultured with high sucrose concentrations and desiccated to low moisture content (MC), so as remove most or all freezable water in the explants prior to liquid nitrogen exposure. During freezing in liquid nitrogen, vitrification of internal solutes takes place thus avoiding lethal intracellular ice crystallization. As whole of the explant is kept intact, high survival rates and rapid growth are observed post rewarming. The generic protocol for cryopreservation of shoot tips by encapsulation-dehydration technique (Figure 2a) is described below:

Dissection of shoot apices: Excise shoot tips (about 2mm), after removing the leaf primordia using a dissecting microscope from the one month old cultures.

Encapsulation: Suspend the excised shoot tips in 3% sodium alginate solution. Dispense individual shoot tips along with the alginate solution into the liquid MS medium supplemented with 100mM calcium chloride, using a sterile pasteur pipette. Allow to stand for 20-30 minutes for complete polymerization. The alginate polymerizes into calcium alginate and forms beads in which the shoot tips get enclosed.

Osmotic dehydration: Remove the encapsulated shoot tips from the liquid medium and suspend in MS medium supplemented with 0.75M sucrose, for 48 hours.

The higher osmoticum of the medium dehydrates the beads, and the concentration of the intracellular solutes increases due to sucrose uptake by the cells.

Air dehydration: To further decrease the moisture content of the explants, remove the beads from the high sucrose medium and place them on sterile petriplates in a laminar air flow for 4-6 hours.

Determination of Moisture content and regeneration per cent**:** Moisture content by oven drying method (ISTA, 1999) and regeneration percentage on optimized regeneration medium of the beads is determined at three different stages.

- Stage 1 – freshly encapsulated shoot tips
- Stage 2 – shoot tips post osmotic dehydration
- Stage 3 – shoot tips post air dehydration

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

Thawing and regrowth: Remove the cryovials from liquid nitrogen after 1 hour and thaw them by placing in a water bath maintained at 40°C for 2 mins. After thawing, transfer the beads to the regeneration medium and incubate in dark for 3 days. Then remove the shoot tips from the beads and subculture on the fresh medium. Observe the survival and regeneration of shoot tips.

Vitrification

Vitrification, encapsulation vitrification and droplet vitrification techniques involve the use of cryoprotectants. Cryoprotectants are the compounds that reduce the freezing point and super-cooling point of water so as to retard ice crystal formation during the process of cryopreservation thus preventing the damage caused to cells by freezing or thawing. DMSO, ethyl glycol and glycerol are most widely used cryoprotectants.

The procedural steps involved are detailed below:

- Explant- Dissection of shoot apices: Collect apical shoot tips (2-3 mm) from the rapidly growing *in vitro* grown 4-6 weeks old plantlets.
- Preculture of excised shoot tips: Culture the sterilized shoot tips on pre culture medium (containing0.3 M sucrose in MS basal medium) and incubate overnight at 25°Cunder 16 h light/8 h dark photoperiod.
- Osmotic Dehydration: Immerse the shoot tips in osmoprotective loading solution for 20 min at 25°C.
- Vitrification with PVS2**:** Following preculture, transfer the shoot tips to 1.8 ml cryovial containing 1 ml vitrification solution PVS2. Incubate for 90 min at 0°C.
- Freezing in liquid nitrogen: Following cryoprotective dehydration with PVS2, plunge the cryovial into liquid nitrogen. Maintain non-frozen controls at 25°C.
- Thawing, unloading and regrowth: 1) Remove the cryovials from liquid nitrogen after 1 hour and thaw them by placing in a water bath maintained at 40°C for 2 mins; 2) Remove the vitrification solution from cryovials with unloading solution, provide 2-3 wash in unloading solution and then incubate the shoot tips immersed in unloading solution for 20 min.; 3) For regrowth, remove shoot tips from the cryovials and blot them dry on sterile filter paper and implant on regeneration medium.; 4) Assess the survival, 10-12 days after cryopreservation by greening of the shoot tips and regrowth by counting the number of shoot tips that had developed leaves; and Record the observations everyday up to 4-8 wks.

Droplet- Vitrification (DV)

Droplet-vitrification is one of the new vitrification-based techniques of cryopreservation, where droplets of cryoprotectant were placed on an aluminum foil strips, into which explants (meristems, shoot tips/buds, nodal segments, somatic embryos, zygotic embryos and cell

suspensions) are placed for LN freezing and rewarming. Droplet-vitrification allows higher rate of cooling and thawing compared to normal vitrification (of about 130^{o}C/min). In recent past, droplet vitrification has been applied to many crops *viz.*, *a*sparagus, apple, banana, cassava, C*hrysanthemum* × *sinense,* date palm, garlic, grapes, mentha, potato, rose, sweet potato, strawberry, taro, tomato, vanilla, yams, etc.

The procedural steps (Figure 2b) involved are detailed below:

- Preparation of explants

  i. Remove shoots from mother cultures and place in the base of a Petri dish.

  ii. Dissect the shoot tips (1–2 mm) from each shoot and place in a pregrowth medium.

  iii. Incubate the petri-dish overnight at at 25°C or lower temperature.

- Cryopreservation

  i. Immerse shoot tips (5–10) into a petri dish with osmoprotective loading solution for 20 min at 25°C (optional) (Pretreatment of explants)

  ii. Transfer shoot tips to petri-dish with PVS2 (duration depends on the species)

  iii. Label the cryogenic vials, fit it in the cryogenic storage box (25-place cryogenic vial storage box) and place in the styrofoam box containing LN. Place sterile aluminum strips to petriplate and evenly place five to eight 5 µL droplets of cryoprotectant solution onto each sterile strip of aluminum foil.

  iv. Place explants into each droplet on the aluminum foil strips (2 min before the end of the PVS2 exposure time).

  v. Plunge the aluminum foil strips with adhering droplets containing the shoot tips in LN till the bubbling stop. After bubbling stops, transfer the strips into a cryo vial in cryogenic storage box (25-place cryogenic vial storage box) placed in a Styrofoam box containing LN. Transfer the vials to a cryogenic storage unit.

  vi. Controls were processed in the same way except that they were not stored in liquid nitrogen and dark.

- Warming, unloading and regrowth

  i. Remove cryo-vial caps and take out aluminum foil strip with explants with a fine forceps and place in petri dish with 10 ml of unloading solution at room temperature and leave it for certain period (depends on the species) for diffusion of DMSO from the tissue.

  ii. Transfer the warmed shoot tips to another petri dish containing filter paper to remove adhered unloading solution.

iii. Place shoot tips on the recovery medium after draining the adhered solution, and kept it in dark for 4 days at 25°C (wrapped with aluminum foil to provide dark condition).

iv. After 4 days, remove wrapped aluminum foil and incubate at 25–26°C under light from cool white fluorescent lamps (50–80 µmol m^2 s^{-1}; 16 h light: 8 h dark).

v. Regrowth is recorded after 4–6 wks. Transfer the recovered shoots to test tubes with regrowth medium.

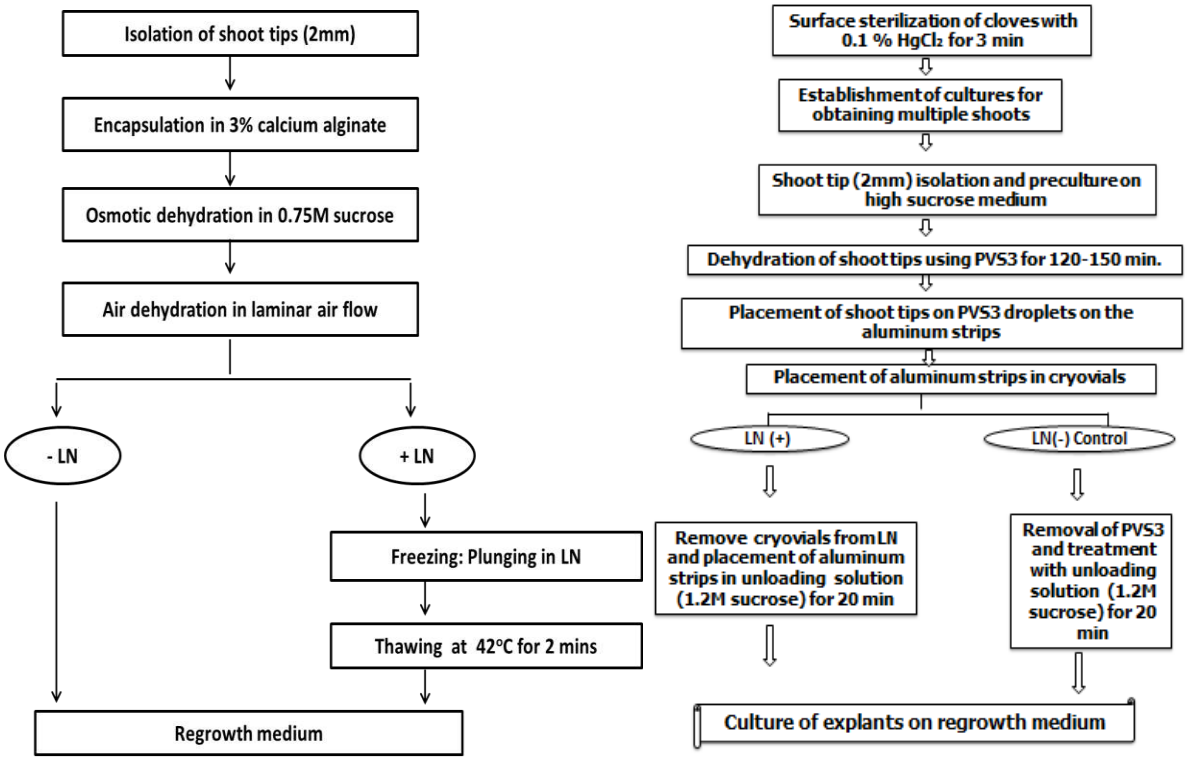

Figure 2: Generic protocol for cryopreservation of shoot tips by; a) encapsulation-dehydration technique, b) Droplet-Vitrification

Prepare in advance-

- Pre culture medium (MS basal medium containing 0.3M sucrose)
- Osmoprotective loading solution: 2 M glycerol + 0.4 M sucrose in liquid MS medium at pH 5.8
- Cryoprotective solutions (PVS2, PVS3)

  PVS2: 30% (3.26 M) (w/v) glycerol, 15% (2.42 M) (w/v) ethylene glycol, 15% (1.9 M) (w/v) DMSO in 0.4 M sucrose in liquid MS at pH 5.8

  PVS3: 50% glycerol + 50% sucrose
- Unloading solution: liquid MS with 1.2 M sucrose at pH 5.8
- Mother cultures: In vitro cultures of mother plants as a source of shoot tips

- Sodium alginate solution (3%) in liquid MS medium excluding halides and containing 0.5 M sucrose, pH 5.8.
- Calcium Chloride solution (100mM) in liquid MS medium without halides, pH 5.8
- Osmotic dehydration solution (0.75M sucrose) in liquid MS medium
- Recovery medium: standardized as per plant species

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Biochemical and Phytochemical Evaluation of Germplasm

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The role of germplasm is well recognized in the improvement of cultivated plants owing to the variability available in these resources for agronomic traits. Primarily the crop breeding efforts were concentrated at improving the crop productivity and thus the traits associated with productivity such as number of tillers per hill, no. of spikes/branches, no. of grains, grain weight, early flowering/crop maturity and tolerance to various stresses have been harnessed to a larger extent. Now the world is witnessing epidemic of degenerative diseases such as alzhiemer, Parkinson, obesity, diabetes, cardiovascular diseases, cancers which are associated with deficiencies of micronutrients, protein and excessive intake of calories and low intake of protective phytochemicals. So now the scientific communities across the world are focussing towards diversifying food basket as well as improving the nutritional profile and phytochemical composition of food crops.

Germplasm diversity contributes to achieve Sustainable Development Goals (SDGs); with respect to food and nutritional security for each and every one; sustainable food systems, lively hood security and healthy vibrant ecosystems. In this context use of the terms evaluation with reference to 'Biochemicals' (biological molecules-proteins, carbohydrates, lipids, nucleic acids, etc.) and 'Phytochemicals' (chemicals of plant origin- alkaloids, cardiac glycosides, flavonoids, antioxidants, etc.) need a clear mention and understanding.

However, use of germplasm for improving nutritive value of crops is very limited owing to non availability of nutritional profile data for germplasm collections and lack of rapid tools for assessing inheritance of traits in breeding populations. Germplasm diversity can lead to nutrient deficiency/sufficiency in diet as nutrient composition between crops and among varieties/cultivars of the same crop can differ drastically. Folic acid and iron are extremely deficient in human population across the world and is one of the most significant factor for anaemia. Folic acid is about 130 µg/100 g in lentil while moth bean has about 350 µg/100 g. Iron content of rice is about less than one mg/100g, while in amaranth seeds is 9mg/100g, this reflects the importance of having different food crops in our daily diets. Similarly huge variability is observed for different traits within the crop species such as protein content ranges in rice (6-15%), maize (5.5-14%), barley (9-17%), chickpea (14-28%), pigeon pea (14-27%), lentil (18-29%), green gram (19-30%), amaranth (7-15%); sweet potato differ in carotenoid content by a factor of 200 or more; similarly bananas have carotene less than 1

µg/100 g to as high as 8,500 µg/100 g. Similarly, wide variability is observed in phytochemical profile of food crops and medicinal plants such as phenols, flavonoids essential oils, and other secondary metabolites. Biochemical and phytochemical profiling data of germplasm resources can be useful for plant breeders in selecting germplasm for targeted traits and use them in improvement for food crops and medicinal plants.

Various methods are used for assessing specific nutrients which involves gravimetric methods (moisture, ash, crude fat, dietary fiber), spectroscopic (total sugars, total carotenoids, total phenols, total phytate, total saponins, total oxalates etc.) chromatography coupled with spectroscopy such as HPLC/UPLC with UV-VIS/fluorescence/mass spectroscopy or GC with flame ionisation detector/mass spectroscopy are used for separation, identification and quantisation of individual molecules like amino acids, sugars, oligosaccharides, polyphenols, flavonoids, carotenoids, organic acids, vitamins, alkaloids, essential oils etc. Minerals can be estimated by colour reactions based spectroscopic methods but they are less sensitive and prone to error. Hence use of atomic absorption spectroscopy (AAS)/ inductively coupled plasma linked to atomic emission spectroscopy (ICP-AES)/ optical emission spectrometry (ICP-OES)/mass spectrometer (ICP-MS) are preferred methods for minerals profiling. In addition to standard wet chemistry based methods spectroscopy techniques including NIR/IR/Raman/XRF/NMR are used for rapid estimation of different nutrients non-destructively.

However, quality of analytical data depends on several factors and influenced because of errors in sampling, sample homogenisation, extraction methods, losses due to incomplete extraction or degradation of analyte during extraction, calibration errors in measuring devices particularly electronic weighing balances, auto-pipettes, spectrophotometers. Inappropriate methods which are not validated often give inaccurate estimations; hence care must be taken in selection on methods. Method must be tested for accuracy, precision and recoveries as well as validated in lab by use of certified reference material (CRM), food reference material (FRM) or in-house quality control (QC) samples.

Sampling is an important to select representative sample, which is true to type of original bulk sample. Such as for grains representative sample can be obtained by mixing grains properly so that all grains are mixed else there is a tendency of small grains to settle down at bottom. When collecting sample from fresh fruits and vegetables longitudinal section from centre is true close representative compared to transverse section.

Sample homogenisation: for samples with less than 15% moisture and oil content homogenisation through cyclone mill is the most appropriate methods as the method is not only rapid but also provides uniformity in particle size of flour besides there is less heating of samples and easy cleaning of mill so less contamination of sample. However for mineral estimation one should choose appropriate accessories and check for any contamination of

metals coming to sample in the process of homogenisation. Samples with medium moisture can be either dried in lyophilizer, vaccum oven, dehumidifier drier and homogenised in cyclone mill, while the samples with high moisture should be homogenised using titanium/stainless steel knife based blender grinder and processed immediately for analysis.

Extraction methods: several options are available for extraction of analyte such as extraction by diffusion in solvents (can be accelerated by shaking, heating, ultra sonication, vacuum invigoration or their combination), Soxhlet extraction, pressurised liquid extraction, super critical fluid extraction are some of them. Most important is check the solubility, recovery and stability of analyte in solvent used. One can check the solubility from scientific literature as well as by testing different solvent combinations, recovery and stability can be confirmed by either testing certified reference material or by spiking the samples with standard.

Calibration of measuring devices: Electronic weighing balances should be checked for calibration every time they are switched on by using certified weights. If error is observed balance must be calibrated as per the manual of balance. Auto-pippets are usually the most common cause of random/systemic error in analysis which could be either due to drying up of grease in piston of pipette or shift in calibration. Thus it is important check the pipettes for these errors. One can test them by measuring the weight of defined volume of water pipetted through these pipettes on pre-calibrated weighing balance. If same deviation is observed every time then it needs calibration where as if deviation is random then pipette needs servicing and calibration.

Testing of UV-Vis spectrophotometers for absorption and wavelength accuracy: International Union of Pure and Applied Chemistry (IUPAC) constituted commission on physicochemical measurements and standards has recommended several reference materials for realization of physicochemical properties in 1976. Of these Copper sulphate solution in aqueous sulphuric acid for testing absorbance accuracy in visible range, potassium dichromate solution in aqueous sulphuric acid for testing absorbance accuracy in UV range can be easily prepared in chemistry laboratory. Similarly solution of Holmium III Oxide in aqueous perchloric acid for testing wavelength accuracy based on peaks of absorption spectra is an easy method.
Selection of appropriate analytical techniques: Before making the selection of analytical techniques analyst is required to assess under-mentioned factors

Ability to conduct analysis: Sample size, reagents, instruments, cost, state of sample

Fundamental characteristics: Precision, accuracy, sensitivity, specificity, detection limit, reproducibility

Personnel concerns: Safety, simplicity, speed

Technique status: Official method, in-house method

Official methods are developed by being comprehensively studied and compared between laboratories. Standardized official method includes, which have been published by AOAC International, American Association of Cereal Chemists and American Oil Chemists' Society. Additional specialized method collections, such as Food Chemical Codex for determination of additives, have also been compiled. In addition to numerous techniques are not listed as official method for the reason that they are relatively new or have not yet been applied to certain types of samples. In these cases, in-house methods may be used if they have been validated. Majorly analytical techniques are grouped into chromatographic, spectroscopic, gravimetric, microbiological assays.

Chromatographic techniques

Chromatography is based on the principle of distribution or partition coefficient of a sample solute between stationary phase; which is always composed of solid phase or layer of a liquid adsorbed on the surface a solid support and mobile phases; which is always composed of liquid or gaseous component. Previously few reliable chromatographic methods were commercially available and most chemical separations were carried out using a variety of techniques including open-column chromatography, paper chromatography, and thin-layer chromatography. However, these chromatographic techniques were inadequate for quantification of compounds and resolution between similar compounds. Chromatographic techniques in common use today in food analysis include gas chromatography (GC), ultra/high performance liquid chromatography (U/HPLC), and supercritical fluid chromatography (SFC). These often serve as a separation method when connected to another instrument such as a mass spectrometer, which serves as the detector

Column chromatography

In column chromatography, the stationary phase is held in a narrow tube through which the mobile phase is forced by gravity. The stationary phase is a solid, generally silica gel or alumina which separates the components of a liquid/ mixture passing through it by selective adsorption on its surface. The various electrostatic forces cause the adsorption between the solute and solvent. Owing to the selective adsorption power of the solid phase, the component ideally will separate at different rates. A more weakly adsorbed compound will be eluted more rapidly than a more strongly adsorbed compound.

Thin layer chromatography

The thin layer chromatography involves the same principles as column chromatography, and it is also is a form of solid-liquid chromatography. In this case, however, the solid adsorbent is spread as a thin layer on a glass plate. A drop of the solution to be separated is placed near one edge of the plate, and plate is placed in developing chamber. The solvent migrates up carrying with it the components of the mixture at different rates. This technique is very rapid and easy for identification of unknown compounds.

Gas chromatography (GC)

GC was introduced in the 1950s and has been applied to a wide range of foods. It is applicable to volatile substances that are thermally stabile; LC and SFC are more appropriate chromatographic methods for analysis of amino acids, peptides, sugars, and vitamins. GC is useful for analysis of nonpolar compounds, although polar compounds may be analyzed if derivatized first. Isolation of the analyte from the sample matrix is particularly important in GC to avoid false responses from matrix degradation products. Headspace methods (including direct sampling of the headspace), distillation, and solvent extraction are often employed. Most common detectors used in food analysis are flame ionization (FID) and mass spectroscopy (MS) The most common food analysis applications for GC involve fatty acids, essential oils profiling, sugars/oligosaccharides, amino acids, alcohols and other volatile compounds.

Ultra/High-Performance Liquid Chromatography (U/HPLC)

HPLC was developed in the 1960s as an improvement over column liquid chromatography and has been used to measure nonvolatile food components. Spectroscopic detectors are often employed. Normal-phase U/HPLC, in which the stationary phase is a polar adsorbent and the mobile phase is a nonpolar solvent, is often used for fat-soluble vitamins and carbohydrates. Reversed-phase U/HPLC, with a nonpolar stationary phase and polar mobile phase, is more popular because of its wider application. Ion-exchange HPLC, with a functionalized organic resin as packing material, is used for detection of inorganic ions and analysis of carbohydrates and amino acids. U/HPLC is currently the most popular food analysis technique (GC is second) and is most used for amino acids, carbohydrates, drugs, lipids, and proteins.

Spectroscopic Techniques

Spectroscopy consists of many different applications such as Atomic Absorption Spectroscopy, Ultraviolet-visible Spectroscopy, Fluorescence Spectroscopy, Infra-red Spectroscopy and Nuclear Magnetic Spectroscopy. Spectroscopy is based on interactions of matter with electromagnetic radiation. Interactions can take the form of absorption and emission, and can be detected by using emission, transmission, and reflection designs.

UV, Vis and Fluorescence

UV and Vis spectroscopy measure absorbed radiation and have been used in food laboratories for many years. A food component that absorbs in the ultraviolet or visible range may be analyzed at its characteristic wavelength in a UV-Vis spectrophotometer, as long as there are no interfering compounds. Fluorescence spectroscopy deals with emitted radiation, and can be three orders of magnitude more sensitive than UV or Vis spectroscopy. Many organic molecules fluoresce, including bacteria and some pesticide residues, making fluorescence spectroscopy an option for detecting food contamination

Infrared (IR/NIR)

Many molecular groups absorb IR light at specific wavelengths in an infrared spectrum, with the fingerprint region of the spectrum leading to positive identification of compounds. Whereas NIR region does not generate fingerprint rather overtones and combinations give slopy spectra which has to be derivatised to higher mathematical orders such as 2^{nd}, 3^{rd} or 4^{th} derivatives. However NIR region helps in quantization of different biomolecules through machine learning techniques and development of prediction models based on actual chemistry data of samples.

Raman spectroscopy

Raman spectroscopy is a complementary technique to IR spectroscopy. IR absorption depends on changes in dipole moment, meaning that polar groups have strong IR responses. Raman scattering deals with changes in polarizability of functional groups, so nonpolar groups produce intense responses. Proteins and amino acids lend themselves to Raman spectroscopy, and carbohydrates, lipids, and minor food components are also examined by this technique.

Atomic Absorption and Atomic Emission

Atomic absorption spectroscopy (AAS) is based on absorption of UV-Vis radiation by atomized minerals, whereas atomic emission spectroscopy (AES) uses the emission of radiation by a sample. Samples must usually be ashed, dissolved in water or dilute acid, and vaporized. In AAS, samples are atomized by nebulizer and burner (flame AAS), or by a graphite furnace (electrothermal AAS). In AES, atomization and excitation can be performed by flame or by inductively coupled plasma (ICP), where samples are heated to over 6000 K in the presence of argon. Both AAS and AES measure trace metal concentrations in complex matrices with excellent precision and accuracy.

Mass Spectrometry (MS)

A mass spectrometer ionizes molecules to produce charged fragments that are separated by size and charge. MS has been used for identification and analysis of complex compounds since the early 1960s. The coupling of separation techniques with MS, which began in the 1970s, has overcome the main analytical problem with chromatographic techniques — namely, ambiguity about the identity of the analyte. MS is frequently used in combination with GC, U/HPLC and ICP. MS techniques have been used to analyze the gamut of food components, including antioxidants, aroma compounds, carbohydrates, drug residues, lipids, peptides and proteins, toxins, and vitamins.

Nuclear Magnetic Resonance (NMR)

NMR is a spectroscopic method in which atomic nuclei oriented by a strong constant magnetic field absorb characteristic frequencies in the radio range. It is nondestructive, do not

usually require sample separation or extraction, and can analyze the interior of a sample. NMR experiments are performed using continuous wave (magnetic field held constant and oscillating frequency varied, or vice versa) or pulse (short time, large amplitude) methods; Low resolution NMR instruments are used for moisture or oil content.

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Molecular Techniques for Germplasm Characterization

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Germplasm characterization is the key to germplasm utilization. Characterization allows discrimination among individuals, group them based on their similarity, construction of core or reference sets, documentation of duplicates, identification of gaps for further collection of novel germplasm for conservation and posterity, genetic stability testing, DNA profiling, hybrid purity testing, identification of valuable germplasm for plant breeding programmes through QTL/association mapping. Germplasm characterization can be achieved through morpho-agronomic, biochemical and molecular characterization. In the present era, molecular techniques are the quickest mode of large-scale characterization and are unaffected by environmental conditions. A number of molecular techniques are available for germplasm characterization which include gel-based separation [Random Amplified Polymorphic DNA (RAPD), Inter Simple Sequence Repeats (ISSR), Amplified Fragment Length Polymorphism (AFLP), Simple Sequence Repeats (SSRs), Sequence Related Amplified Polymorphism (SRAP), Start Codon Targeted Polymorphism (SCoT) etc.); capillary based fragment separation (AFLPs, SSRs) and sequence level separation [Single Nucleotide Polymorphism (SNPs), Genotyping By Sequencing (GBS), Next Generation Sequencing, Resequencing] for differentiation/identification of individual accessions/ genotypes/ varieties. For gel-based and capillary based fragment separation, Polymerase Chain Reaction (PCR) is the basic requirement. DNA extraction is the first step for all the DNA and PCR based molecular techniques used for germplasm characterization (Annexure I).

Following are the basic steps of the methodology used for PCR and gel based molecular techniques employed in germplasm characterization:

- DNA extraction, purification and quantification
- PCR
- Gel electrophoresis
- Gel imaging
- Data scoring and statistical analysis

DNA extraction, purification and quantification

DNA extraction is a technique of isolating DNA from biological samples. The three basic

steps of DNA extraction are: 1) Lysis of cell (cell wall/lipid bilayer outer membrane) to release DNA 2) Separation/precipitation of DNA from proteins, lipids, sugars, secondary metabolites (polyphenols etc.) and inorganic ions present in the cell; 3) Purification. A number of plant DNA extraction procedures are available based on the usage of different chemicals/reagents, each performing a specific function. Cetyl trimethylammonium bromide (CTAB) is most common method (Saghai-Maroof, 1984) used for isolating DNA from plants. And DNA can be extracted from any plant tissue viz. seedlings, leaf, root, seeds etc. Following are the functions of different reagents used for isolating DNA using CTAB method:

Cetyl trimethylammonium bromide (CTAB) is a detergent and is used for isolation of DNA from plant tissues. It disrupts the membranes and binds to the polysaccharides, under the high-salt conditions, and thus removes them from the solution.

β-mercaptoethanol (β-ME) breaks the disulfide bonds between the cysteine residues and denatures proteins and helps in the removal of the tannins and polyphenols.

EDTA is a cation chelating agent and chelates the Mg++ ions which are necessary in activity of nucleases, thus keeping the DNA safe during extraction.

Sodium acetate breaks up into Na+ and [CH3COO]- and the positively charged sodium ions neutralize the negative charge on the PO3- groups on the nucleic acids, making them much less soluble in water.

Chloroform helps phenol in the denaturation of proteins and proper separation of aqueous and organic phase. The nucleic acids and other contaminants such as salts, sugars, etc. will remain in the upper aqueous phase, while the proteins and hydrophobic lipids will partition into the lower organic phase.

Enzyme proteinase K is also used for denaturation of proteins, which however again is denatured and removed via phenol chloroform extraction.

PVP (polyvinyl pyrrolidone) is used to remove polyphenols.

Isoamyl alcohol reduces foaming and stabilizes the interphase (coagulated proteins) between the aqueous and organic phase.

Ethanol/Isopropanol is used for precipitation of nucleic acids. Hydrogen bonding between Ethanol/Isopropanol and water molecules decreases the number of water molecules for hydration of nucleic acids and causes precipitation. Cold ethanol/Isopropanol results in quick precipitation and high yield by slowing down the activity of nucleases.

70% alcohol is used to remove the excess of salts.

RNAase A helps in the digestion of RNA so as to get pure DNA.

FLOWCHART of DNA EXTRACTION

Seedling (100mg)

↓

Crush using liquid nitrogen in a pestle-mortar

↓

Transfer powdered sample into eppendorf tube containing 750µl of preheated CTAB buffer including 2µl of β-ME, mix it thoroughly

↓

Incubate at 65˚C for 30 to 45 min. with intermittent mixing

↓

Open the lid and add equal volume of Chloroform : isoamylalcohol (24:1)

↓

Mix it for 5 to 8 min. gently by forming ∞ in air

↓

Centrifuge at 10000 rpm for 15 min.

↓

Take out upper aqueous layer in another Eppendorf tube

↓

Add 750 µl of chilled isopropanol and mix gently

↓

Precipitates of nucleic acids will be visible or keep it at -20˚C for 2 hrs for precipitation

↓

Centrifuge at 10000 rpm for 10 min. and decant the supernatant without disturbing pellet

↓

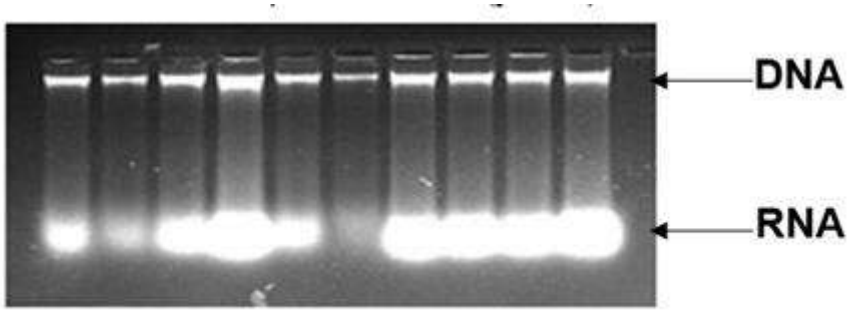
Add 1.0ml of 70% ethanol, keep it for 10 min. and decant the supernatant (repeat it twice)

↓

Air dry pellet for overnight

↓

Add 500 µl of TE buffer (pH 8.0)



Unpurified DNA

FLOWCHART of DNA PURIFICATION

Add 2 µl of RNAase A to unpurified nucleic acid solution (500 µl) and incubate at 37°C for 1h

↓

Add equal volume of Phenol:Chloroform:Isoamylalcohol (25:24:1) and mix it gently

↓

Centrifuge at 12000 rpm for 10 min

↓

Collect the supernatant in a new Eppendorf tube

↓

Add equal volume of Chloroform: Isoamylalcohol (24:1) and mix it gently

↓

Centrifuge at 12000 rpm for 10 min.

↓

Repeat above step twice

↓

Add 1/10 volume of 3M sodium acetate (pH 5.6) and 2.5 times (v/v) chilled ethanol (100%)

↓

DNA will precipitate

↓

Centrifuge at 12000 rpm for 10 min. and decant the supernatant without disturbing pellet

↓

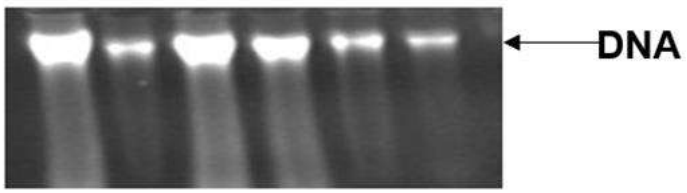
Add 1.0ml of 70% ethanol, keep it for 10 min. and decant the supernatant (repeat it twice)

↓

Air dry pellet for overnight

↓

Add 200 µl of TE buffer (pH 8.0)

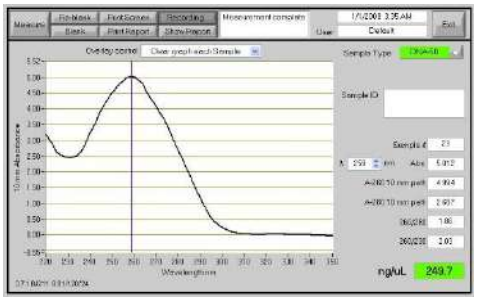


Purified DNA

DNA Quantification (Nanodrop Microvolume Spectrophotometer)

Nanodrop microvolume technology uses a sample retention system and is based on the surface tension properties of the sample being measured so as to form a liquid column. For proper column formation, it is essential that the sample makes contact with the upper and lower optical measurement surfaces. Sample heterogeneity or liquid column breakage may be the cause of inaccurate or non-reproducibility of results.

- Pure DNA typically yields a 260/280 ratio of ~1.8
- Pure RNA typically yields a 260/280 ratio of ~2.0
- Pure DNA typically yields a 260/230 ratio of 1.8-2.2



This how Purified DNA Spectra looks like!

FLOWCHART OF DNA QUANTIFICATION

Use lint-free lab wipe to clean the upper and lower optical surfaces of Nanodrop sample retention system with deionized water

↓

Open the NanoDrop software to select the Nucleic Acid Application

↓

Dispense 1 µl of buffer onto the lower optical surface and lower the lever arm and select "Blank" in the Nucleic Acid Application

↓

Clean both optical surfaces with a clean, dry, lint-free lab wipe

↓

Choose the appropriate constant for the sample that is to be measured

↓

Dispense 1 µl of DNA sample onto the lower optical pedestal and close the lever arm

↓

Select "Measure" in the application software to measure DNA concentration

↓

Assess the sample quality by checking 260/280 or 260/230 ratios in combination with overall spectral quality

PCR (Polymerase Chain Reaction)

PCR was developed by Kary Mullis in 1983 to make lots of copies of a particular segment of DNA. PCR consists of three basic steps: 1) Denaturation of DNA to separate double stranded DNA in to single strands, which is generally carried out at 94 to 96°C; 2) Annealing at temperature which enables primers to bind/attach to DNA and it is 37°C for decamer primers, RAPD; varies from 47 to 61°C for ISSR primers and SSR primer pairs; 3) Extension is done at 72°C during which Taq DNA polymerase adds DNA bases to the single strand of DNA in 5' to 3' direction. These three steps are repeated 20 to 40 times to get the number of copies of desired DNA sequence. Annealing temperature* is calculated from melting temperature (Tm) and is generally kept 5ºC lower than Tm of the primer.

Melting temperature (T_m) = 4 * Number of G or C + 2 * Number of A or T (°C) in a oligo/primer

Primer: short fragments/stretches (18-25 nucleotides) of single stranded DNA, which are designed to bind to a specific piece of template DNA during PCR

Taq DNA Polymerase: a thermostable enzyme which synthesizes new strands of DNA complementary to target sequence during PCR

dNTPs: A(Adenine), T(Thymine), G(Guanine), C(Cytosine) are the dNTPs, which are added as complementary bases to synthesize new strand of DNA

$MgCl_2$: Mg^{2+} is a cofactor for Taq DNA polymerase and influences its enzymatic activity

PCR reactions are carried out in a PCR Thermal Cycler and following protocols are used for setting up PCR reaction.

PCR component concentrations and temperature conditions (SSR):

PCR components	25 µl reaction mix (1R)	Final Concentrations
dH_2O	15.8 µl	
Template DNA (20 ng)	2.0 µl	40ng
10x PCR assay buffer (without $MgCl_2$)	2.5 µl	1X
$MgCl_2$ (25 mM)	1.5 µl	1.5mM
dNTP mix (10 mM)	0.5 µl	0.2mM
Taq DNA polymerase (5U)	0.2 µl	1.0U
Primer (10.0 µM) (Forward and Reverse)	1.25 µl each	0.5µM each

Thermocycling Conditions	RAPD/ISSR/SSR
Initial Denaturation	94ºC x 3 min.
Denaturation Annealing* Extension	94ºC x 30 sec. ---ºC x 30 sec. 72ºC x 1 min. (35 cycles)
Final Extension	72ºC x 4min.

Thermocycling Conditions	SSRs
Initial Denaturation	94ºC x 3 min.
Denaturation Annealing Extension	94ºC x 30 sec. 62ºC x 30sec. ↓ 0.7ºC/cycle 72º C x 1 min. (10 cycles)
Denaturation Annealing Extension	94ºC x 30 sec. 55ºC x 30 sec. 72ºC x 1 min. (35 cycles)
Final Extension	72ºC x 4min.

DNA Gel Electrophoresis

This technique is used for the separation of DNA molecules. On applying an electric field to the gel matrix, negatively charged DNA molecules will migrate towards positively charged electrode and get separated based on size.

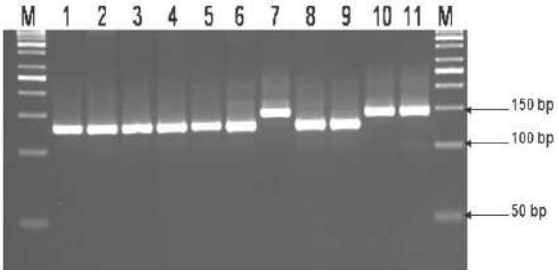
Low percentage agarose gels (0.3–0.8%) is used for separating large DNAs, while small DNAs are separated using higher percentages (2–4%) of agarose gels. Tris, borate and EDTA (TBE) buffer is good for separating smaller fragments and for separation of large size products, Tris, acetate and EDTA (TAE) is used. There are certain advantages of using TBE over TAE due to following reasons:

1) TBE is less prone to overheating than TAE, so good for long runs 2) TBE gives nicer bands compared to TAE 3) fragments with <2kb size better resolves in TBE than TAE.

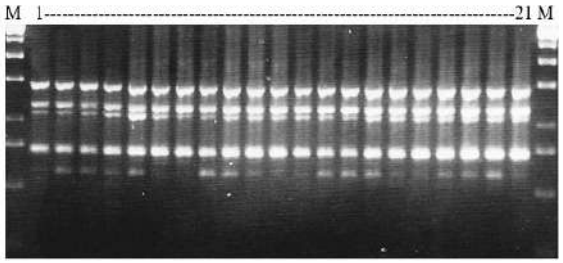
After completion of PCR amplification, 2.0µl of 6x loading dye is added to each PCR tube. Agarose gel (3.0 to 3.5% metaphor agarose for SSRs; 1.4 to 1.6% regular agarose for RAPD, ISSR etc.) in 1X chilled TBE buffer with 15 µl ethidium bromide (1mg/ml) per 100 ml of gel volume is prepared. Size standard/ molecular weight marker (M) depending upon the size of the PCR products to be separated, is also run in one or two lanes for sizing of the PCR products, which are to be separated in agarose gel. Electrophoresis is carried out at the rate of 4-10 V/cm, cm refers to the length of the gel. Electrophoresis is carried out at 150 V for 1 hours followed by run at 100 V till the bromophenol blue dye travelled less than 2/3rd the length of gel for a medium size gel. The resolved PCR amplification products are visualized under UV light on a UV- Transilluminator. The gel is photographed using an Imaging System.

Ethidium Bromide or EtBr intercalates DNA and on exposure to UV, it will fluoresce with an orange color, and helps in DNA visualization.

Loading Dye has two functions, one it is used as tracking dye (Bromophenol Blue and Xylene Cyanol) to visualize colourless DNA in the gel and secondly to provide density to the sample so that it will sink into the bottom of each well in the gel.



Gel Image of SSR in finger millet



Gel Image of RAPD in finger millet

Gel scoring and Statistical Data Analysis

The amplification products are scored across the lanes comparing their respective molecular weights. Each band is treated as one RAPD/ ISSR/SSR marker. Scoring of bands is done from photographs. Homology of bands is based on distance of migration in the gel. Presence of a band is scored as "1", absence of a band as "0" and missing data as "9".

In case of SSRs bands are treated as alleles, so alleles of different sizes are scored and recorded. And different genetic diversity parameters can be calculated using the formulas given in Statistical Formula section.

	SSR Locus 1	SSR Locus 2	SSR Locus 3	SSR Locus 4
Sample 1	180	210	160	201
Sample 2	186	218	170	209
Sample 3	190	230	166	211
Sample 4	180	210	166	201
Sample 5	180	210	178	201
Sample 6	196	218	178	211
Sample 7	200	230	186	201
Sample 8	196	224	178	209
Sample 9	186	210	160	209
Sample 10	200	218	186	201

SSR allelic data record sheet

Marker	Major.Allele.Frque	SampleSize	AlleleNo	GeneDiversity	Heterozygosity	PIC
rr1	0.3939	67.0000	4.0000	0.6890	0.0606	0.6282
rr2	0.4925	67.0000	5.0000	0.6001	0.0000	0.5218
rr3	0.9403	67.0000	2.0000	0.1123	0.0000	0.1060
rr4	0.4179	67.0000	5.0000	0.7114	0.0896	0.6641
rr5	0.5952	67.0000	7.0000	0.5881	0.0952	0.5457
rr6	0.7719	67.0000	2.0000	0.3521	0.0351	0.2901
rr7	0.7424	67.0000	3.0000	0.4042	0.0152	0.3547
rr8	0.8657	67.0000	2.0000	0.2326	0.0000	0.2055
rr9	0.3806	67.0000	5.0000	0.7284	0.0299	0.6829
rr10	0.4851	67.0000	4.0000	0.5898	0.0299	0.5053
rr11	0.3507	67.0000	6.0000	0.7339	0.0149	0.6890
Mean	0.5851	67.0000	4.0909	0.5220	0.0337	0.4721

Output of Power marker data for SSR markers

Following are the basic genetic diversity data analysis input and output format as per most popularly used software: NTSYSpc. Following is the Input data in excel for NTSYSpc :

These are scot primer morinda diversity							
20 primers were run							
1	12	7	1	9			
	Amarkan1	Amarkan2	Amarkan3	Dhar	Kathiwad1	Kathiwad2	Kathiwad3
rr1	1	0	1	1	1	1	0
rr2	1	0	1	1	0	1	1
rr3	1	0	0	0	0	0	0
rr4	1	0	1	1	1	1	1
rr5	1	0	1	0	1	0	1
rr6	0	1	0	1	1	1	1
rr7	1	0	1	0	0	0	0
rr8	0	1	0	1	1	1	1
rr9	0	0	0	1	0	0	9
rr10	0	0	0	0	0	1	9
rr11	1	1	1	1	1	1	9
rr12	0	0	0	0	0	1	9

The genetic associations between accessions/genotypes are evaluated by calculating the Jaccard's similarity coefficient for pair wise comparisons based on the proportion of shared bands produced by the primers using the program NTSYS-pc. (Rohlf, 2000). Cluster analysis is performed by agglomerative technique using the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) method by the SAHN clustering function of NTSYS-pc. Relationships between the accessions are portrayed graphically as dendrograms.

How Jaccard's similarity coefficient is calculated?

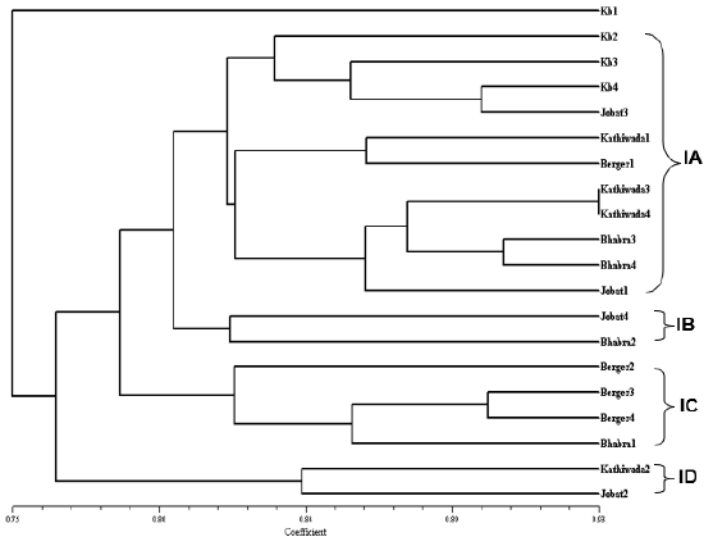
Presence/ absence of a band in ith and ith taxa	**Individual B**	1	0
Individual A	1	a	b
	0	c	d

Where, 1= band present; 0= band absent; Jaccard's coefficient: J= a/a+b+c

Jaccard's Similarity Matrix

	GE4	GE47	GE70	GE99	GE113	GE128	GE145	GE149
GE4	1							
GE47	0.588							
GE70	0.656	0.759						
GE99	0.636	0.677	0.611					
GE113	0.543	0.606	0.611	0.588				
GE128	0.618	0.767	0.735	0.688	0.541			
GE145	0.548	0.643	0.613	0.895	0.382	0.548		
GE149	0.767	0.677	0.629	0.559	0.618	0.719	0.618	1

UPGMA Cluster (Dendrogram)



Statistical formula used in molecular characterization

- Expected heterozygosity/ gene diversity = $(1- \sum pi^2)$, where pi is the frequency of the ith allele
- Resolving power of a primer (Rp) = ∑Ib = sum of Band Informativeness of all bands produced by a primer and Band Informativeness (Ib) = 1-(2 x |0.5 –p|), where p is the proportion of genotypes containing the band.
- Marker Index (MI) = is product of expected heterozygosity/ gene diversity (Hes)/PIC and effective multiplex ratio.
- Effective multiplex ratio (EMR) = nβ= Effective multiplex ratio (E) is defined as the product of the total number of fragments per primer (n) and the fraction of polymorphic fragments(β).
- Minimal number of markers needed to identify a set of genotypes (n) = In X / [In (1/1-D)], where 'X' is the number of unique genotypes, 'D' is the genetic diversity over the loci and n is the number of loci analyzed.
- Number of genotypes that can be identified (X) = $[1/(1-D)]^n$, where X is the unique genotypes, D is the genetic diversity over loci.
- Probability of chance identity (CIp)= $[XD]^n$, where XD is average similarity index among genotypes and 'n' is the average number of amplified products per genotype.
- Polymorphism Information content (PICi) = [2fi (1- fi)], where i is marker fragment, fi is the frequency of the marker fragments which were present, while (1-fi) the frequency of marker fragments which were absent. PIC (for dominant markers) was averaged over the fragments for each primer.
- Proportion of polymorphic loci (P) = Number of polymorphic loci/ total number of loci.
- Average number of alleles per locus (A) = sum of all alleles divided by total number of loci.
- Mean diversity He is calculated by averaging the gene diversity (Hes) values over all loci and ranges from 0 to 1.
- Effective number of alleles (Aes) = $1/ \sum pi^2$, where pi is the frequency of each allele observed across all loci.

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Liu K, SV Muse (2005) PowerMarker: An integrated analysis environment for genetic marker analysis. *Bioinformatics* 21: 2128–2129.

Peakall R, PE Smouse (2006) GenALEx 6: Genetic analysis in Excel. Population genetic software for teaching and research. *Mol Ecol. Notes.* 6: 288-295.

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Rohlf FJ (2000) NTSYS-pc Numerical Taxonomy and Multivariate Analysis System Version 2.1. Exeter Publishing Setauket, New York.

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Yeh FC, RC Yang, T Boyle, ZH Ye, JX Mao (1999) POPGENE, Version 1.32: The user friendly software for population genetic analysis; Molecular Biology and Biotechnology Centre, University of Alberta: Edmonton, AB, Canada.

Some of the freely available statistical software's for genetic data analysis

Arlequin: http://cmpg.unibe.ch/software/arlequin3/

GDA: https://phylogeny.uconn.edu/software/

GenAlEx : http://biology.anu.edu.au/GenAlEx

Popogene: https://sites.ualberta.ca/~fyeh/popgene_download.html

Power Marker: http://statgen.ncsu.edu/powermarker/index.html

Structure v 2.3: http://web.stanford.edu/group/pritchardlab/structure.html

Annexure I

Solutions, Chemicals and reagents used for DNA extraction

1. Liquid Nitrogen
2. DNA Extraction buffer: 20 g CTAB, 81.9 g Sodium chloride, 40 ml EDTA (0.5M stock) and 100 ml of 1M Tris is added in distilled water and the volume is made to one liter. The solution is autoclaved before use. 0.2 % β- Mercaptoethanol was added after autoclaving.
3. Tris: Cl buffer (1M): 121.1 g of Tris salt is dissolved in distilled water and the volume is made to one liter. The pH is adjusted to 8.0 using concentrated HCl. The solution is autoclaved before use.
4. Ethylene Diamine Tetra Acetic acid (EDTA, 0.5M): 186.1 g of EDTA is dissolved in sterile distilled water. The pH of the solution is adjusted to 8.0 using 1N NaOH. The volume is made to one liter using sterile distilled water and the solution is autoclaved.

5. Isopropanol (100 %)
6. Sodium Acetate solution: 30.75 g sodium acetate is dissolved in sterile distilled water, pH is adjusted to 5.6 with glacial acetic acid and volume made to 50 ml. The solution is autoclaved and stored till use.
7. Chloroform: Isoamyl alcohol (24: 1) mixture: 96 ml of chloroform is mixed with 4 ml of isoamyl alcohol. It is stored in amber colored bottle.
8. 70% ethanol: 70 ml of absolute ethanol is mixed well with 30 ml of sterile water and stored in a stoppered bottle till use.
9. Phenol: Chloroform: Isoamyl alcohol (25: 24: 1): 100 ml of phenol saturated with 10mM Tris HCl and 1mM EDTA at pH 8.0 is added to a mixture of 96 ml Chloroform and 4 ml Isoamyl alcohol. The mixture is mixed well before use and is stored in amber colored bottle.
10. RNase A solution: 10 mg/ml of RNase A solution is prepared in sterile distilled water. The solution is heated at 100ºC for 15 minutes to inactivate any DNase present and then stored at –20º C.

DNA dissolution buffer

Tris: EDTA (TE) buffer (10mM Tris: 1 mM EDTA, pH 8.0): 10 ml of Tris (1M) buffer, pH 8.0 and 0.2 ml of 0.5 M EDTA, pH 8.0 is mixed with sterile distilled water and the volume made to 100 ml. The buffer is autoclaved before use.

Gel Electrophoresis

Agarose gel (1.4 %): 3.78 g agarose is added to 270 ml 1X TBE buffer, the contents are mixed thoroughly and boiled for 2-5 minutes to dissolve the contents. The mixture is cooled to 55ºC. The molten gel is casted in a gel tray with comb to produce wells.

Ethidium bromide (1mg/ ml): 10 mg of ethidium bromide is dissolved in sterile water and volume made to 10 ml. The solution is stored in amber colored bottle at lab temperature.

Tris: Boric acid: EDTA (TBE) buffer (10X stock) solution: Dissolve 108 g Tris and 55 g Boric acid in 900 ml distilled water. Add 40 ml 0.5 M EDTA (disodium salt) (pH 8.0). Adjust volume to 1 litre and store at room temperature.

24
Plant Genetic Resources Informatics – Practical Exercises

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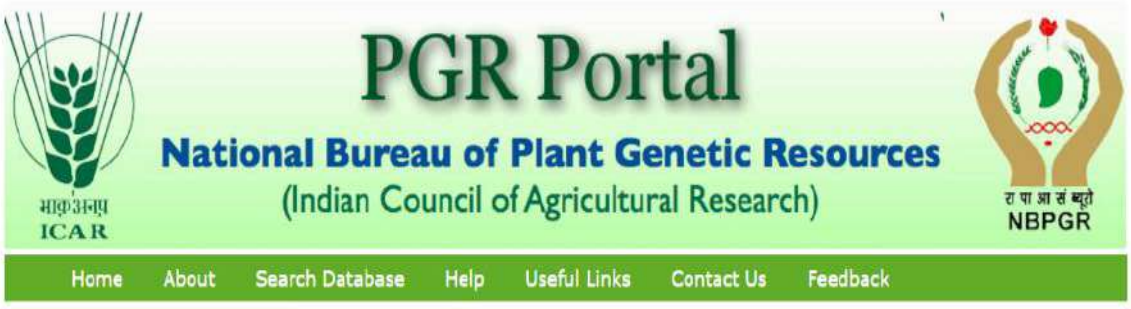
A number of database applications have been developed under PGR informatics like PGRPortal, Passport Information Management System (for both IC and EC), PGRMap, National Herbarium of Cultivated Plants (NHCP), Crop Wild Relative Portal, PGR Clim, Germplasm Registration Portal etc. Few applications are web based while others are intranet application (for internal use on NBPGR Local Area Network only). Following are four exercises for hands-on experience on four of these applications. All applications are accessible through www.nbpgr.ernet.in → PGR Databases.

Practical Exercise 1: PGR PORTAL http://pgrportal.nbpgr.ernet.in/



Access PGR Portal using URL: pgrportal.nbpgr.ernet.in

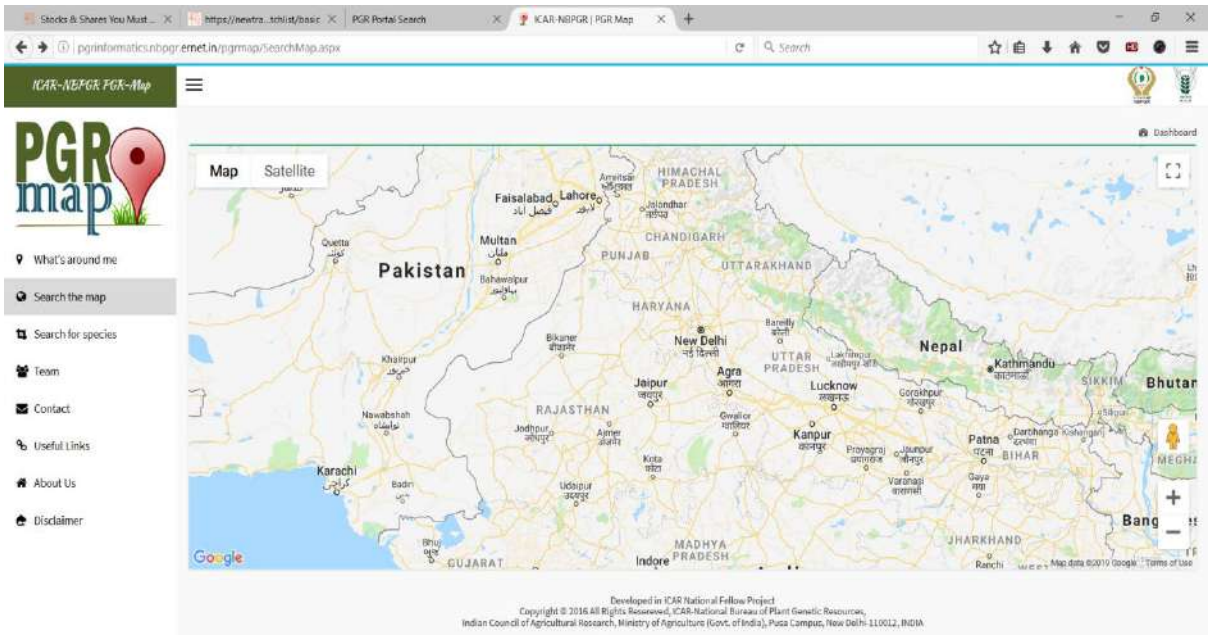
1. Click on 'Passport (Simple)'
   a. Find all accessions with crop name 'Okra' and varieties like 'Pusa'
   b. Search all 'Wheat' accessions collected from Uttarakhand between 01/01/2012 and 31/12/2015 with Biological Status as 'Released Variety'
   c. Search all exotic accessions of Wheat imported from 'Mexico' during last 10 years.
   d. Search all Chilli accessions collected by a collector with collector number like 'RSR/SH'
   e. Search all landraces of Tomato imported from Thailand

2. Click on 'Passport (Free Text)
   a. Search all Mustard with species as '*nigra'*
   b. Search all '*juncea'* species of Mustard with Cultivar Name as 'Lai' or 'Lai Patta'
   c. Extend above query b) with condition where biological status is 'Improved cultivar'
   d. Are there any exotic (imported) '*Brassica'* accessions in the Gene Bank?

e. Are there any 'Elite lines' of *Setaria* available that are collected from Bihar?

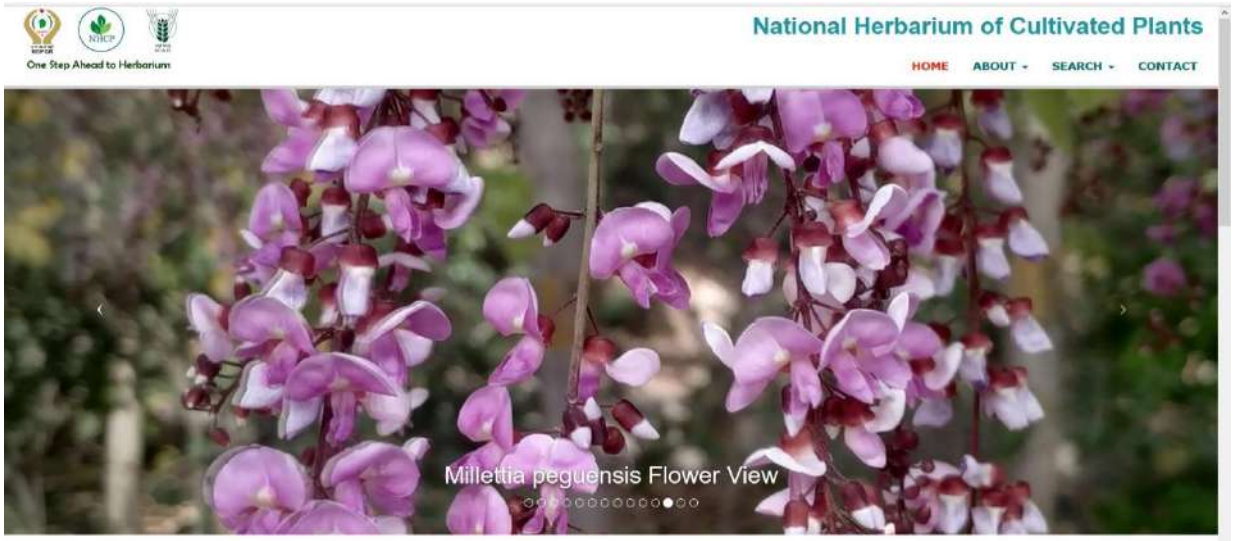
3. Click on Characterisation/Evaluation link
   a. Select the crop as 'Brinjal' and view graphical output of any five descriptors of your choice one by one.
   b. List all accessions of Okra where Fruits per Plant are between 15-30 and Fruit length between 1.5 cm to 8.0 cm

Practical Exercise 2: PGR MAP http://pgrinformatics.nbpgr.ernet.in/pgrmap



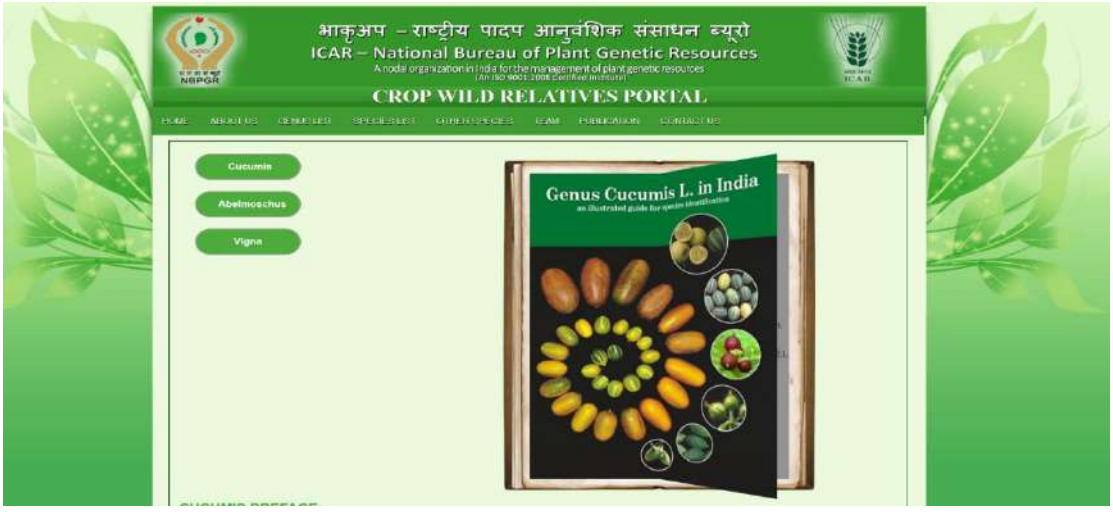
1. Access PGR Map application using URL: http://pgrinformatics.nbpgr.ernet.in/pgrmap
2. Click on 'What is around me' and click 'Share Location' button if a new small window pops-up followed by 'Search here' and see the collections of various crop groups made around your current location.
3. Click on 'Search the map' and found all millets collected from Bikaner (Rajasthan) and its surroundings
4. Search all the Greater Yam collections from Thiruvananthapuram, Kerala
5. Which Oilseeds have been collected from a location in Gujarat neighbouring Pakistan
6. Search all locations from where Tobacco has been collected, is there any from Arunachal Pradesh?
7. How will you find the entire 'Black gram' collected from Balaghat, Madhya Pradesh and its availability in the National Gene Bank?

Practical Exercise 3: E-Herbarium www.nbpgr.ernet.in:8080/nhcp/



1. Access NHCP application using URL: http://www.nbpgr.ernet.in:8080/nhcp/ or by clicking on www.nbpgr.ernet.in → PGR Databases → PGR NHCP
2. Click on 'Search' and tell how many Total Specimen details are available?
3. Search how many crop wild relatives of *Triticum, Sorghum, Abelmoschus* and Pearl millet are available respectively?
4. Search all samples of Poaceae family. Can you view the herbarium picture of Sorghum bicolor?
5. Search all samples of *Abelmoschus palianus* through Advance Search and view their pictures, enlarge (resize) the picture and evaluate picture quality.
6. Advance Search how many Solanum samples in Solanaceae family are available in National Herbarium. Extend your search with *pimpinellifolium* species of *Solanum* genus.

Practical Exercise 4: CWR Portal pgrinformatics.nbpgr.ernet.in/cwr



1. Access Crop Wild Relatives application using URL: http://pgrinformatics.nbpgr.ernet.in/cwr/ or by clicking on www.nbpgr.ernet.in → PGR Databases → Crop Wild Relatives
2. Click on the Menu → Genus List and view booklets for Cucumis, Abelmoschus and Vigna by clicking on the respective buttons one by one.
3. Click on 'Species List' How many total species details are available?
4. Select *Cucumis callosus* and view Species Feature details
5. Find the distribution of *Abelmoschus Manihot* on the Map. Click on various dots appeared on the map for detailed information about the selected sample.
6. Switch to the Satellite view. Do you feel any difference?
7. Try to map the distribution of *Cajanus cajan* in the state of Uttar Pradesh.

25

Plant Genetic Resources Management and Utilization: An Ethos of Indian Traditional Agriculture and Civilizations

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The Indian Subcontinent being one of oldest landmass settled after major geological events, bringing the landmasses of both the hemispheres to present continental picture and being situated at the tri-junction of three realms of global biogeographical zones, namely, Afro-tropical, Indo-Malayan and Paleoarctic, offered great ecological diversity, starting from the temperate high-altitude regions of the Himalayas, the Central Peninsular Highlands and Plateaus, the Thar Desert of north-western plains, the tropical rainforest of Western Ghats, and the diverse forest types of Eastern Ghats along the coast. These diverse ecologies provided conditions for the adaptation of many organisms originating from both the hemispheres and diverse ecologies, including plant species and continued with further evolution of species over time and space making it one of the 12 MegaCenter's biodiversity. It is very rich in floristic diversity, representing around 12% of global diversity, housing 47,513 plants species, including species of bacteria. Of these, about 20,141 taxa are of angiosperms with 17,926 species belonging to 2,991 genera and 251 families, approximately 7% of the described world species (Singh, 2016).

Because of diverse, but comparatively congenial conditions, Indian Subcontinent has inhabited a large amount of ethnic diversity, consisting of both the indigenous races and those moved from other parts of the world as settlers looking for greener pastures with better living. It has been considered to inhibit several indigenous or migrated races, such as Caucasoid (Aaryn), Dravidians, Mongoloid, Negroid, and Australoid; 420 Tribal communities, 227 ethnic groups spread over 5000 forest villages, and 75 primitive tribes, several of which are under threat and at the verge of extinction.

In search of better living from wild/nomadic life Indian Subcontinent has been witness to human evolution from hunter-gatherer stage to practice of domiculture, to settled agriculture. The interactions between ethnic and migrating races, tribes, communities and the prevailing biodiversity and landscapes resulted in domestication and adaption economically important organism, including plant species of both indigenous (rice) and exotic species (maize, sorghum) coming from both Old and New world. This process led to settled agriculture and more genetic diversity in cultivated species. Subsequently, it led to evolution and establishment of several civilizations, such as Harappan Civilization renamed as Indus-Saraswati Civilization (Misra, 1994; Gupta, 2001) or Saraswati River Civilization (Kalyanaraman, 2001) and Gangetic

Civilization as component of larger *Vedic* civilization or culture, with predominant agriculture-based economy. As per the recent studies, this dynamic process of origin and evolution agriculture continued for millenniums, predominantly at 21 Agricultural Biodiversity Heritage Sites, spread over the Indian Subcontinent with adaption of economic species into cultivation in suited regional ecologies and brought further revolution in the growth of agriculture and genetic diversity. Ecologically, Indian agriculture has been classified to 21 agro-ecological zones with rich genetic diversity for adapted species.

The present Indian agriculture deals with cultivation of around 811 plant species and harbors more than 900 wild relatives of these cultivated species (Singh *et al*., 2013). Vevilov, 1937; Zeven & De Wet, 1982 respectively estimated 166-171 cultivated species originating from Hindustani Center, while Singh *et al*., 2013 recently estimates domestication of 215 species. Another, around 600 exotic crop species were adapted from different parts of the worlds, starting from ancient pre-Columbian times to date, further revolutionizing Indian agricultural biodiversity. In addition, 1,532 edible plants for food, 9,500 ethno-botanical species and 7500 ethno-medicinal species have been documented.

Agrobiodiversity

Agrobiodiversity refers to variation in agroecosystems, agriculture related plants, animals, fish, insects, microbe, avian etc. They are fundamental to the fulfilment of basic human needs like food, fodder, fibre, fuel, medicine etc., and are 'keystone' components of agrobiodiversity dealing with genetic variation found in the domesticated species, which directly or indirectly essential to sustain humankind. Thus, agrobiodiversity includes, diversity species of interest, their wild relatives and species that are part and/or supportive of agroecosystems.

Plant Genetic Resources (PGR)

Conventionally PGR refer to genetic variation/diversity (germplasm) of actual or potential value that exist among individuals or group of individuals belonging to a cultivated species and its wild relatives. It is key for the genetic improvement of cultivated species and is carried as genetic material/information found in reproductive or vegetative propagating material of plants, such as, seeds, tissues, cells, pollen, DNA molecule etc. containing the functional unit of heredity that can be utilised in crop improvement program.

Tenets of Indian Heritage/Traditional Agriculture Promoting Management and Use of PGR

They are based on *Vedic* beliefs (Figure 1) preaching- Let the good and knowledge come from all sides and love, affection and worship mother nature for environmental peace, survival of earth and co-existence of all living organism. They are dynamic, evolving with time and space. Over time they have been integrated into social and religious beliefs. Therefore, has capacity of integrating knowledge with indigenous knowledge for further advancement of technology.

Started with Sarswati Valley or Harppan Civilization and peaked during Aryan/*Vedic* period. Some key tenets-

- Culture was born and nourished in the forest
- Water, earth, plant, animal, human and the Devin came together in the image of Goddess *Prithivi*
- "Unless man can learn to take some interest and delight in beauty and diversity of the world for its own sake, and unless he can see some use in things not apparently useful" things will not go well
- "We must live for something besides making a living. If we do not permit earth to produce beauty and joy, in the end it will not produce food either".
- It is the responsibility of human beings that they in partnership of God should establish prosperity and wellbeing of the earth.
- Plants possess a soul. There are god and goddesses
- Plants are repeatedly mentioned in Indian religious literature, no ceremony is complete without use of some sacred plant
- Cult of tree worship
- Trees are abode of spirits (home)
- Whole ritual was laid for the felling of trees and for image making for purposes of worship.

ॐ द्यौः शान्तिरन्तरिक्षं शान्तिः पृथिवी शान्तिरापः शान्तिरोषधयः शान्तिः । वनस्पतयः शान्तिर्विश्वेदेवाः शान्तिर्ब्रह्म शान्तिः सर्व शान्तिः शान्तिरेव शान्तिः सा मा शान्तिरेधि ॥ ॐ शान्तिः शान्तिः शान्तिः ॥ — यजुर्वेद ३६:१७	Om. May peace radiate there in the whole sky as well as in the vast ethereal space everywhere. May peace reign all over this earth, in water and in all herbs, trees and creepers. May peace flow over the whole universe. May peace be in the Whole Universe. And may there always exist in all peace and peace alone. Om peace, peace and peace to us and all beings!

Figure 1: Vedic hymn reflecting the belief

Indian Agriculture Traditional Practices Promoting Management and Use of PGR

Gurkul System of Education: Promoting documentation and research

In ancient times Rishi Muni's had their Ashrams (schools) in deep forest. Study of nature, including biodiversity used to be an important component of the curriculum. This led to study of prevailing flora, properties of plant species and identification of economically important plants related to food, fiber, fuel, medicinal potential, etc. This resulted in documentation of large number of plants along with their properties at different stage human evolution making India one of the important centers of knowledge about plant diversity and properties. Consequently, the ancient scriptures from Indian civilization for the first-time reports' documentation plant species with associated knowledge. Sixty-seven species are mentioned in *Rigveda*, 81 in *Yajurveda* and 290 in *Atharvaveda*. *Ayurveda* is credited with the documentation of the plant species with their properties in the three legendary/ authoritative classical texts- Charak Samhita (700 BC) for 1100 plant species; Sushruta Samhita (200 years BC), for 1270 plant species; and Astang Hridayam (AD 700) 1150 plant species (Singh *et al*., 2016). Vrikshayurveda of Surapala (1000 AD) describes the properties and cultivation practices of 170 plants (Sadhale, 1996).

Sacred Plants: *Promoting conservation and use of useful plant species diversity*

Following the basic principle of *Vedic* culture preaching respects for nature, led to identification of multipurpose plants and tradition of planting and protecting the sacred trees like banyan, peepal, bel (*Aegle marmelos*) etc. It dates to Indus valley culture. Bodhi trees are associated with the name of sages who got knowledge meditating under these trees. Identifying plants with the deities was another Aryan tradition. Plants auspicious for flowers or the wood or any other part were used for the sacred fire ceremony, *Homa* (an important component of traditional agriculture for creation of diseases and pest free environment). Garuda Purana mentions of ritual on use of plants and the annual calendar for propagation and offering. Plants revered for life-saving role (Bishnoi's).

S*acred Groves*

Respectful attitude towards mother nature and forest, led to development of basic principles for management of biodiversity in general and forest. Forest were considered sacred, property of village gods. Sacred groves, small patches of <5 h to hundreds of hectares were protected to keep the vegetation unchanged. They often involved with regeneration of forest, organised by the local communities under forest work plans for commercial extraction of goods, such as timber, firewood, forage and other produce. It led to conservation of regional biodiversity, forest management, regulation of cultivation of sacred and economic plant species. Probably it originated in biodiversity rich Meghalaya (A recent study on the diversity of vascular plants in three sacred groves of the Jaintia Hills recorded about 395 species belonging to 250 genera, and 108 families, comprising pteridophytes, gymnosperms and angiosperms), and extended to

Northeast Hills region, Aravalli Hills, Chand and Bastar area in Central India, Konkan and Malabar region in Western Ghats. They are integral to surrounding agricultural settlements.

Bagichi concept

The extension of sacred grove concept at rural areas in villages led to development of *Bagichi* concept to support conservation and maintenance of local biodiversity, particularly of sacred plants used in worship and those with medicinal and aromatic properties. This concept is common to north Indian rural areas, where most villages has an extended garden of local flora and economic plants, facilitating conservation and use. They also had a temple, well, wrestling ring, dharmshala, community centers for social activities, hermit house, etc.

Jhum*: *slash and burn cultivation promoting perpetuation of economic species

It was a step forward from domiculture (raising of diverse species to meet diverse needs in a plot) in agriculture and progress towards settled agriculture from hunting and gathering stage. It involves slash and burning for clearing of forest lands followed by cultivation of mono (rice in Arunachal) or multiples crops around the base crop (Malabar). It is prevalent in Northeast region, Eastern Himalayas, Brahmaputra Valley, Meghalaya, Northeast Hills region. Also, prevalent in Koraput region with the name of "*Podu"* and '*punamvalal*' or *punam* in Malabar region. Some practices of cultivation involve cultivation of multiple (15 to 30 species) crops on the same plot of land (*Swidden* farming involving women of *Angami*s). It has pprogressed to tree-based system in Meghalaya, which is more remunerative, besides promoting conservation and use of wide spectrum of crops diversity.

Homestead farming systems or home/backyard gardens

One of the most ancient systems, it includes practice of cultivation of economic plant species in home/backyard gardens or near habitation. It is most prevalent in Western Ghats. Uses the principle of multistoried intercropping upper layer (trees), second (Pepper, etc.), third (Banana, etc.), basal (ginger, grass, etc.) in Malabar region, matching the natural structure of tropical rain forest. Often combined with livestock, poultry rearing interacting synergistically. Provide nutritional security, fodder, extra income and crop residue for manuring. They are self-contained, need-oriented systems, and are predominantly organic, with biological controls for insect pest and diseases with the use of botanicals such as tobacco decoction, neem extracts, etc.

On-farm maintenance breeding through selection and conservation PGR

Indian Farmers has always been improving the inherited crop diversity through rigorous selection in the standing crops, collecting seed for next sowing. Thereby restricting genetic degradation of open pollinated varieties. This reflects about their knowledge regarding pollination through air. This process in other crops helps in generating genetic (clonal) diversity.

Informal exchange of seed among neighboring farmers and relatives

Again, based on the knowledge of cross pollination, Indian farmers wish to see good crop(s) all around the agroecosystem or the region. They multiply and store seed for informal exchange of quality seeds (superior varietal seeds) with neighbors and relatives. This may help in maintaining vigor in out-crossing crops. Thereby helping conservation and enhancement of genetic diversity.

Development of farming systems promoting conservation of PGR of diverse species

Polyculture involving multiple crops

Revolve around a major crop. The major crop can be a perennial horticulture crop, example coconut or an annual crop, example rice. Promotes synergistic interaction between various biotic components, harnessing the benefit accrued from such interactions and conserving their genetic resources

Mixed cropping

Involve annual crop species. May included short, medium, and long duration crop species to harness interactive benefits and relay harvest. Example *Baranaaja* (12 grains) practiced in Central Himalayas. Cereals and legumes, example millets and groundnut (fast growing & tall millets provide protection). Cereals and others, example wheat and mustard. Cereals and vegetables, example millets and cucurbits. Annual crop and floricultural crops, example marigold on borders to facilitate biological control of nematodes. Crop with tall crop, such as *Dhaincha* (*Sesbania bispinosa*), work like a fence, providing protection.

Crop rotation systems

Evolved during *Vedic* period for effective control of weeds, pests, diseases, by reducing build-up; to improve or maintain soil fertility; check soil erosion; division of labor to spread the workload family labor; mitigate the risk of weather changes; and increase the net profit. May include Crop-Fallow Rotations (periodic resting/close season & beneficial residual effects of certain crop). Crop-Crop Rotations (taking benefit of residual effects of biotic interactions)- Leguminous crops are grown alternatively with wheat, barley, and mustard. Cereal and leguminous crops: pigeonpea – wheat; cereals – chickpea; Cereals-oilseeds-cereals: Millet followed by castor; Pearl millet in rotation with sorghum, groundnut, cotton, minor millet, castor, etc. In dry and light soils pearl millet is often rotated with a pulse-like moth or mungbean

Local biodiversity-based agroforestry system

It includes integration of local trees, shrubs, bamboos, etc. in field cultivation. In India, it is believed to have been initiated during *Vedic* era. *Khejri* or *sami* (*Prosopis cineraria*), *aswattha* (*Ficus religiosa*), *palasa* (*Butea monosperma*) and *varana* (*Crataeva roxburghii*) has been mentioned in ancient literature of *Rig Veda*, *Atharva Veda,* and other ancient scriptures. The tree species that are known as, either nitrogen-fixer or efficient recycler of the available

nutrients to improve soil fertility. Examples are *Acacia*, *Albizia*, *Alnus*, *Erythrina*, *Faidherbia*, *Ficus*, *Inga*, *Parkinsonia*, *Pilhecellobiuin*, *Prosopis*, *Robinia* and *Sesbania* and The multipurpose trees, such as *Areca catechu*, *Azadirachta indica*, *Butea moonosperma*, *Dalbergia sissoo*, *Tectona grandis*, *Artocarpus* spp., *Casuarina equisetifolia*, *Mangifera indica*, *Phoenix dactilifera*, *Pterocarpus santalinus* (red sandal), *Ceiba pentendra*, *Leucaena leucocephala*, *Grevillea robusta*, *Bambusa arundinacea*, *Erythrina variegata*, *Syzygium aromaticum* and *Zizuphus mauritiana* for economic gains. Vary from shifting cultivation to complex home-gardens to high-density complex multistoried homesteads gardens in humid tropical lands of the Western Ghats, and regular sparse stands of trees on farmlands, example: *Khejri* (*Prosopis cineraria*) in arid zone of western India, Alder (*Alnus nepalensis*), in North-east Hills.

Mixed farming involving other components agriculture/agrobiodiversity

This practice involving other component of agriculture to help furthering management and use of genetic resources of components suited to diverse agroecosystems. It may involve livestock, such as cattle, buffalo, sheep, pigs or poultry, ducks, etc., or aquatic life, fish, turtle, crab, etc. For example, Homestead Garden in Malabar region involve following components- 1. uplands mixed with different crops, 2. uplands with crops and livestock, 3. uplands associated with adjoining lowlands including crop only, 4. uplands associated with adjoining lowlands including both crops and livestock, and 5. uplands with adjoining backwaters with crops, poultry, livestock, and agro-based industries (salinity tolerant rice genetic diversity).

Biodiversity based forest management systems conserving wildlife

Indian Subcontinent also has tradition of growing native economically important plants in remnant habitat areas and/or forest margins. This help in restricting the degradation of lands. Economically important local species can provide additional produce/income to local communities/farmers for diverse use. Also, it helps in conservation of local biodiversity and ecology of the area. Some common species used in such efforts are grasses such as *Bothriochloa* spp., and *Eragrostis curvula*, trees like *Azadirachta indica, Acacia* spp. Many more such strategies might have played role in evolving *Sacred groves* and *Bagichi* concept and to enhance eco-system functions, such as carbon sequestration, reduced toxicity.

Seed Storage: For emergency and disaster management

Short-term seed storage for next sowing. Community seed storage in temple etc., and long-term storage in temple top Kalsh (at *Shree Padmanabhaswamy Temple,* Thiruvananthapuram). The pots were made of five metals with such precision that anything stored cannot be spoiled for 12 years. May be that's why the grains are refilled after 12 years on the occasion of *Kudamuzhugu Vilzha*. In a situation of natural calamity, say flood or something else, these seeds are intact and used when things start getting back to normal.

Agricultural advantages

- Ensure availability of diverse genetic resources and products supporting human life and requirements, such as herbal medicine
- Economic pest and disease management through biological control methods
- Reduced fertilizer and pesticides and fungicides dependence
- Provided zero-residue of toxicity, avoiding health hazards and pollution
- Ensuring cleaner environment and water
- Improved pollination, in case of cross-pollinated and often cross-pollinated crops

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Characterization of Plant Genetic Resource for Genomics-assisted Crop Improvement

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Abstract

Development of genetically improved a/biotic stress tolerant and high-yielding crop cultivars is crucial to ensure their optimal yield and productivity and thus global food and nutritional security amidst current changing climatic conditions. However, most of these yield and stress tolerance traits are complex and quantitative in nature and regulated by multiple genes. This implicates an essentiality of developing novel advanced breeding strategies along with traditional breeding strategies for quick quantitative dissection of aforesaid complex traits in crop plants. To accomplish this, the conventional genetics and breeding approaches complemented with diverse genomics-assisted breeding strategies appear quite promising for crop genetic enhancement. Therefore, the future prospects of crop breeding are more inclined towards integrated use of various structural, functional and comparative genomics coupled with classical genetic inheritance studies for rapid dissection of complex yield and stress tolerance traits through genetic and association mapping as well as for genetic improvement of crops. Tremendous technological advances in sequencing and other high-throughput sequence- and array-based genotyping assays in last decade have provided much needed impetus to molecular genetics and breeding. Draft whole genome, resequencing as well as global transcriptome information for many important crop plants are now publicly accessible. This sequence information has since been used to develop vast range of genomic resources including molecular marker repository for large-scale genetic analysis in crop plants. Similar to advancement in sequencing and genotyping technologies, significant progress has also been made in the area of high-throughput phenotyping which has accelerated the precise phenotypic characterization of huge core and mini-core crop germplasm accessions available at different national as well as international germplasm repositories. Availability of high-quality genome-wide genotyping and phenotyping information of natural germplasm accessions and mapping/mutant populations as expected led to identification of many important genes/QTLs (quantitative trait loci) associated with vital agronomic traits using various traditional as well as recently developed advanced genetic mapping and integrated genomic approaches. These identified genes/QTLs have already been exploited to understand the complex genetic architecture of quantitative traits and in translational genomic applications for developing high-yielding, climate-resilient varieties in many important crop plants by marker-assisted breeding. The current chapter in-depth reviews and discusses recent progress and future prospects on

plant breeding vital for genetic enhancement of important food crops. Through revisiting the major landmark research in crop plants, the knowledge gained from successful endeavours especially pertaining to genomics-assisted crop improvement can be translated for their genetic enhancement in order to sustain global crop productivity.

Plant genomic and transcriptomic resources

The advancement of sequencing technology enables the scientist community to uncover the hidden information specifically at genome, transcriptome and epigenome level in a cost and time effective manner. The sequencing efforts have traditionally been performed using first generation Sanger sequencing technology. In the early 2000s, the next generation sequencing technologies NGS); Roche 454/FLX Pyrosequencer, ABI SOLiD and Illumina Solexa Genome Analyzer have been discovered, which expedites the whole genome sequencing efforts in many plant genomes either individually or along with Sanger sequencing. One of the most constraints in sequencing the genome is the presence of highly repeat-rich region in the genome. To overcome the problem associated with sequencing the repeat-rich regions in the genome, third generation sequencing technologies such as Pacific Bioscience (PacBio) that provide long (more than 5 kb) single molecule reads are expected to improve the sequencing and assembly of repeat-rich plant genomes. The whole/draft genome sequencing efforts using the first-generation Sanger sequencing-based clone-by-clone and/or whole genome shotgun (WGS) and next-generation (NGS)-based WGS approaches have been accomplished in diverse crop genotypes. Using these approaches, till date around 100 plant genomes have been sequenced including cereals (rice, wheat, maize, sorghum, barley and *brachypodium*), legumes (lotus, *medicago*, chickpea, pigeonpea and soybean), vegetables (tomato, potato, melon, cucumber, hot pepper and watermelon), fruits (banana, grape, papaya, apple, peach, chinese plum, strawberry and sweet orange) and fibre crops (foxtail millet, mustard, flax, sesame and cotton) (Michael and Jackson 2013). These complete/draft plant genome sequencing efforts have generated enormous genomic sequence resources, including structurally and functionally annotated protein-coding genes and transcription factors. Next generation sequencing also enables to re-sequence the genome of diverse crop genotypes leading to generate a huge number of genomic sequence resources for structural, functional and comparative genome analysis. The genome sequences also shade light on the evolutionary aspect of the sequenced plants, thus facilitating to identify the genes underlying the domesticated traits.

The macro-array analysis [suppression subtractive hybridization (SSH) and cDNA-AFLP (Amplified fragment length polymorphism)] and array-based whole genome transcriptome profiling [microarray chips, serial analysis of gene expression (SAGE) and massively parallel signature sequencing (MPSS)] and currently the whole genome NGS-based transcriptome sequencing/RNA sequencing (RNA-seq) assayed in different vegetative and reproductive tissues during developmental stages of diverse crop genotypes under normal growth and stress-induced conditions are underway. These sequencing efforts have expedited the generation of

large-scale ESTs (expressed sequence tags), full-length cDNA sequences and unigenes (NCBI GenBank, http://www.ncbi.nlm.nih.gov) as well as numerous transcript sequences including differentially expressed transcripts encoding the known/candidate genes (NCBI, GEO database) globally. The enormous genomic and transcriptomic sequences are available with on-line public databases [NCBI (http://www.ncbi.nlm.nih.gov), EMBL (http://www.embl.de), EBI (http://www.ebi.ac.uk), DDBJ (http://www.ddbj.nig.ac.jp), The Institute for Genomic Research (TIGR) (http://rice.plantbiology.msu.edu), Phytozome (http://www.phytozome.org) and TAIR (http://www.*Arabidopsis*.org)] for unrestricted use. Transcriptome atlas for several crop plants including rice and medicago have been generated to pave the way of understanding the complex gene expression networks at different developmental stages of crop plants. For instance, a cell type transcriptome atlas that includes 40 cell types from *japonica* rice shoot, root and germinating seed at several developmental stage have been developed (Jiao *et al.* 2009). Another atlas of reproductive development in Nipponbare has also been developed (Fujita *et al*. 2010). In *indica* rice (IR64), transcriptomic dynamics across various stages of vegetative and reproductive development have been studied using whole genome microarray profiling (Sharma *et al*. 2012). In chickpea, to track the tissue specific gene expression, a transcriptome dynamics across several tissues, including flower bud, pod, root, shoot have been developed (Garg *et al*. 2011). In *medicago*, a gene expression atlas that provides a global view of gene expression in all major organ systems of this species, with special emphasis on nodule and seed development, have been developed (Vagner *et al*. 2008).

Integrated genomics-assisted breeding strategies to delineate functionally relevant molecular tags governing agronomic traits

To expedite the identification of potential trait-influencing genes, QTLs, alleles and haplotypes through genomics-assisted breeding for crop genetic enhancement, the use and/or integration of strategies like genetic/QTL mapping and association analysis have been considered. To achieve those, the large-scale validation and high-throughput genotyping of sequence-based robust genic and genomic SSR and SNP markers in natural germplasm collections (association panel) and advanced generation bi-parental mapping/mutant populations and their further integration/correlation with multi-locations/years replicated field phenotyping data have been initiated in many crop plants using the modern high-throughput genotyping assays and phenotyping platforms.

Plant genetic resource rich in trait diversity

The germplasm resources, including cultivated varieties, breeding lines, landraces, wild accessions representing diverse agro-climatic regions of the world available for diverse crop species have been stored efficiently in different National and International germplasm repository centres including International Rice Research Institute (IRRI), International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), National Bureau of Plant Genetic

Resources (NBPGR) and International Centre for Agricultural Research in Dryland areas (ICARDA) and National Plant Germplasm System-United States Department of Agriculture (NPGS-USDA). For example, about 102547 accessions of *Oryza sativa*, 1651 accessions of *O. glaberrima* and 4508 accessions of 22 wild ancestors of rice (McNally *et al.* 2009) and more than 20000 germplasm lines of chickpea (Gaur *et al.* 2012) are now available at these centres. According to FAO reports (2012-13), about 856158, 235688, 466531, 40820 and 98285 accessions of wheat, sorghum, barley, pigeonpea and potato, respectively are now accessible in different germplasm resource centres developed around the world for their large-scale phenotyping and genotyping. Considering the difficulties involved in genotypic and phenotypic characterization of these huge set of available germplasm resources of crop species, efforts have been made currently to constitute the core and mini-core collections in several crops by identifying the largest amount of genetic diversity with a minimum number of accessions. By the efforts of International institutes like IRRI, ICRISAT and USDA, a set of 932, 242, 211, 238, 146 and 184 germplasm lines belonging to the core/min-core collections of rice, sorghum, chickpea, pearl millet, pigeonpea and groundnut have been constituted from 55908, 37904, 16991, 21594, 13632 and 15490 accessions available for these respective crop species (Upadhyaya *et al.* 2001, 2002; Zhang *et al.* 2011) utilizing both marker-based genotyping and phenotyping strategies and different precise statistical measures. These readily available core/mini-core germplasm resources of many crop plants have been phenotyped at different geographical locations (multi-environment) for several years in field for diverse important agronomic traits including yield component and stress tolerance traits. Based on phenotypic and genotypic characterization of germplasm lines, genotypes contrasting for different agronomic traits including yield component and stress tolerance traits have been selected and utilized as parents for generation of advanced bi-parental and back-cross mapping populations, RILs (recombinant inbred lines), NILs (near isogenic lines) and DHs (double haploids) in many crop plants. Some of these selected contrasting accessions have been induced with different mutagens, including EMS (ethyl methanesulfonate) and γ-ray and generated mutant lines of diverse crop genotypes to identify functional mutation sites for qualitative and quantitative trait regulation. For instance, about 66891 EMS, MNU (N-methyl-N-nitroso urea), sodium azide and γ-ray irradiated mutant lines (Wu *et al.* 2005; Till *et al.* 2007) of rice and 10000 EMS-induced mutant lines of chickpea are currently available (http://tilling.ucdavis.edu; http://www.iris.irri.org) for mining of novel trait-influencing alleles for their genetic improvement.

High-throughput phenotyping and marker genotyping

To expedite the process efficient and precise phenotyping, a larger set of natural/mutant and mapping populations generated for many crop plants have recently been phenotyped for diverse complex yield, and stress component traits using automated modern high-throughput phenotyping and E (environmental)-typing platforms (Xu *et al.* 2012, Mir *et al.* 2012). For

high-throughput and precise phenotyping of complex quantitative traits in many crop plants, an International Plant Phenomics Network (IPPN) has been developed (Clark *et al.* 2011).

Rapid developments in various high-throughput genotyping assays have further elevated the utility of molecular markers in various crop improvement applications. High-throughput genotyping of sequence-based informative markers (SSRs and SNPs) in a larger set of core/mini-core germplasm lines, mapping populations and mutant collections have been hasten currently using various array-based and next-generation sequencing assays such as TILING array, Illumina GoldenGate and Infinium assays, Fluidigm dynamic array, KASP (KBioScience Allele-Specific Polymorphism) profiling, MALDI-TOF, Affymetrix GeneTitan array, Reduced Representation library (RRL) and Genotyping-By-Sequencing (GBS) assay. The automated fragment analyzer, MALDI-TOF, Illumina GoldenGate and Infinium assays and KASP profiling have been considered much advantageous and utilized widely for high-throughput genotyping of prior mined SSR and SNP markers in many crop plants, including rice and chickpea (Parida *et al.* 2012; Gaur *et al.* 2012; Hiremath *et al.* 2012). The GBS assay has now been extensively utilized for simultaneous genome-wide discovery and genotyping of SNPs in diverse plant species (Poland *et al.* 2012; Morris *et al.* 2013; Sonah *et al.* 2013; Spindel *et al.* 2013). It thus expedited the mining of novel functional allelic variants and their large-scale validation and genotyping at whole genome level for constructing high-resolution genome map as well as in efficient QTL and trait association mapping of diverse small and large genome crop plants.

Identification and mapping of QTLs/genes

Realizing the advantages of sequence-based robust SSR and SNP markers, high-throughput genotyping of these markers in advanced generation bi-parental mapping populations enabled to construct high-density genetic linkage and functional transcript maps and hasten the process identification and mapping of genes/QTLs associated with agronomic traits in many crop plants. For instance, about 4861, 388, 122 and 530 QTLs associated with yield component and stress (abiotic and biotic) tolerance traits have been identified and mapped in rice, wheat, chickpea and tomato, respectively (**Figure 1**) by utilizing inter-/intra-specific high-density SSR and SNP marker-based genetic linkage maps (http://archive.gramene.org/qtl, http://solgenomics.net/search/phenotypes/qtl, Varshney *et al.* 2013; Suresh *et al.* 2014). The marker-based genetic linkage map constructed and trait-specific QTLs identified and mapped on chromosomes of different crop species have now become a resource for generating more high-resolution integrated genetic, physical and genome maps (Varshney *et al.* 2014) as well as fine mapping and map-based cloning/positional cloning of trait-influencing genes/QTLs. These approaches traditionally been proved to be the most powerful tools for gene isolation and dissection of the complex quantitative yield and stress tolerance traits in crop plants. For constructing SSR and SNP marker-based high-density and integrated genetic linkage/transcript maps in several crop species, high-throughput next-generation whole genome and

transcriptome sequencing have been successfully applied at present (Huang *et al.* 2009; Xie *et al.* 2010; Gaur *et al.* 2012; Hiremath *et al.* 2012). The constructed high-density genetic linkage maps have been integrated with sequence-based physical map and improved the resolution and accuracy of trait-specific genes/QTLs identification (Wang *et al.* 2011) by additional genome/gene-based fine-mapping and thus significantly expedited the process of fine mapping and map-based gene isolation and positional cloning of genes/QTLs in crop plants. Application of NGS based genotyping approaches have now made possible to accelerate the identification and mapping of genes underlying the major as well as minor QTLs. Recently, a rapid method called "QTL-seq" has been developed for mapping of major genes/QTLs by whole genome NGS based resequencing DNA two bulked populations (Takagi *et al.* 2013). To identify candidate genes encoding transcripts and its regulatory sequences (transcription factors) involved in expression of quantitative traits in crop plants, the "genetical genomics"/ "expression genetics" integrating the genetic or QTL mapping with transcript profiling have been developed (Emilsson *et al.* 2008). The transcripts showing differential expression either by traditional macro-/micro-arrays or next-generation transcriptome sequencing to the whole genome and their correlation with QTL mapping enabled to identify 'expression QTLs' (eQTLs) involved in the *cis*- and *trans*-trait regulation.

Trait association mapping

The candidate gene-based association mapping and genome-wide association study (GWAS) relying on the large-scale genotyping of informative SSR and SNP markers and robust field phenotyping information of naturally occurring core/mini-core germplasm lines (association panel) have now considered to be an effective approach for identification of major and minor genes/QTLs and alleles regulating the simple qualitative and complex quantitative traits in crop plants (Zhao *et al.* 2011; Li *et al.* 2011). The candidate gene-based association mapping by utilizing the genotyping information of SNPs in different coding and regulatory sequence components of genes among a trait-specific association panel have significance to identify genes/QTLs controlling yield contributing and stress tolerance traits in crop plants (Fan *et al.* 2009; Mao *et al.* 2010; Kharabian-Masouleh *et al.* 2012; Parida *et al.* 2012; Negrao *et al.* 2013). With the availability of huge high-throughput genome-wide SSR and SNP marker-based genotyping information of germplasm lines belonging to an association panel, the GWAS has now become a routine approach for high-resolution scanning of the whole genome to identify target genomic regions including genes/QTLs (major and minor QTLs) associated with traits of agricultural importance in many crop species (Huang *et al.* 2010, 2012; Zhao *et al.* 2011). However, the integration of trait association mapping with traditional bi-parental linkage/QTL mapping have recently been implemented to identify functionally relevant robust genes/QTLs for dissecting the complex quantitative yield and stress component traits in crop plants. It is quite evident from the study of *GS3* (Wang *et al.* 2011) and *GS5* (Li *et al.* 2011) genes/QTLs for grain size trait regulation, metal transporter gene regulating aluminium tolerance (Famoso

et al. 2011) in rice and acid phosphatase gene governing low-phosphorus tolerance in soybean (Zhang *et al.* 2014). An integrated approach by combining candidate gene-based association mapping with QTL mapping, differential transcript profiling and LD (linkage disequilibrium)-based gene haplotyping have been developed recently to identify functionally relevant transcription factor genes and QTLs controlling 100-seed weight/seed size in chickpea (Kujur *et al.* 2013, 2014). The trait-influencing molecular tags identified in diverse crop plants have significance to be utilized for genomics (marker)-assisted crop improvement program.

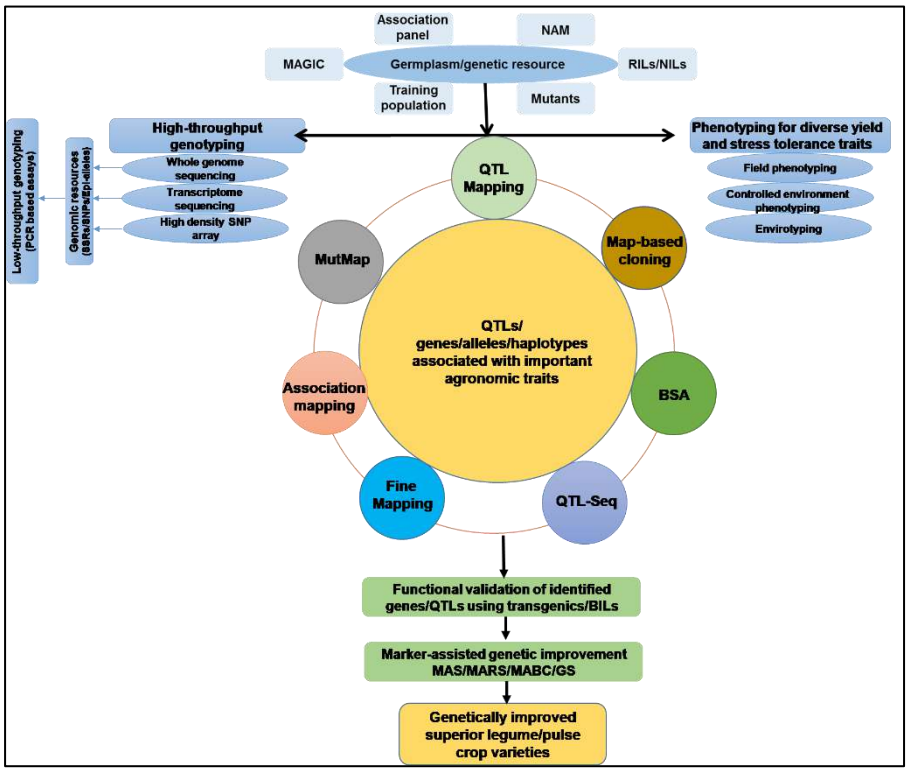
Genomics-assisted crop improvement

The functionally relevant molecular tags regulating the qualitative and complex quantitative traits, identified individually and/or integrated approach of traditional bi-parental linkage/QTL mapping, fine mapping/positional cloning, whole genome and candidate gene-based association mapping and genetical genomics/eQTLs have now been utilized for introgression, combining and pyramiding into selected crop genotypes of interest through traditional and advanced genomics-assisted breeding approaches to develop superior high-yielding stress tolerant crop varieties. The introgression of functional natural genetic variations and favourable genes/QTLs/chromosomal segments identified from a larger set of germplasm lines including landraces and wild species particularly for yield and stress component traits have been transferred into the cultivated genetic background for their crop improvement by employing approaches like introgression lines (ILs), advanced-backcross QTL (AB-QTL) analysis, association genetics and multi-parent advanced generation intercross (MAGIC) population (Tian *et al.* 2006; McCouch *et al.* 2007; Tan *et al.* 2008; Huang *et al.* 2012). The molecular tags showing major effects on qualitative and quantitative trait regulation have now been transferred into diverse crop genotypes for their genetic enhancement through marker-assisted selection (MAS) including marker-assisted back-crossing (MABC)/ marker-assisted foreground and background selection. The genetic improvement of Basmati rice for yield, quality and resistance to bacterial leaf blight and blast diseases has been performed by pyramiding the multiple genes/QTLs through MAS and MABC (Joseph *et al.* 2004; Sundaram *et al.* 2008; Gopalakrishnan *et al.* 2008; Singh *et al.* 2011). The sub-mergence tolerance in swarna using the *Sub1* QTL (Septiningsih *et al.* 2009), drought tolerance in Nagina22 rice using *DTY1.1* QTL (Vikram *et al.* 1999), and drought tolerance and biotic stress tolerance in ICC 4958 and C 214 chickpea by using QTLs associated with root architecture and fusarium and ascochyta blight resistance (Varshney *et al.* 2013, 2014) have been enhanced through MAS. It suggested the implications of MAS for introgression of trait-influencing major effect molecular tags into selected crop genotypes for their genetic enhancement.

The complications in genetic background effects/epistasis and linkage drag of QTLs as well as minor effects of minor and major QTLs/genes on complex trait regulation have impeded the use of traditional MAS (QTL-MAS) approach for the genetic enhancement of crop plants for complex quantitative traits. To overcome these intricacies, many novel advanced genomics-

assisted breeding approaches such as marker-assisted recurrent selection (MARS), MAGIC and genomic/genome-wide (haplotype) selection have been emerged currently in transferring and pyramiding the favourable alleles of minor effect genes/QTLs controlling the complex quantitative traits for genetic enhancement of crop plants for yield and stress tolerance (Meuwissen *et al.* 2001; Jannink 2010; Chia and Ware 2011). The available traditional and novel genomics-assisted breeding approaches provide clues for quantitative dissection of complex trait regulation and thus have potential to expedite the complex trait genetic enhancement studies in diverse crop species.

A significant efforts have been made for functional validation and understanding the molecular mechanisms/biological significance of potential trait-regulatory genes, alleles and haplotypes by developing over-expression and knockout/knockdown (genome/gene-edited) transgenics as well as t-DNA and transposon-mediated mutant complementation assays in crop plants. The integration of genomics-assisted breeding and transgenics have now proven to be the most promising approach for genetic enhancement of crop plants by manipulating diverse complex yield-contributing and stress-responsive traits. The diverse aspects specifically pertaining to genomics, epigenomics, proteomics, metabolomics and genomics-assisted breeding can be applied individually and/or an integrated manner at different time points of study for effective genetic and molecular dissection of complex quantitative traits in crop plants. The inputs obtained from these combined strategies can be used further in various marker-assisted genetic improvement studies for developing stress tolerant high-yielding varieties in diverse crop plants **(Figure 1)**.



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