VIDYASAGAR UNIVERSITY

Memo No. VU/R/Noti/366/2020 Dated 19/03/2020

Conservation: Strategies for conservation

(*in-situ* & *ex-situ*)

Lecture for 4th Semester Special Paper

(BOT 402A) :

Angiosperms Taxonomy and Molecular Systematics

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21st March, 2020

Cryopreservation

Why preservation is important?

Until two decades ago the genetic resources were getting depleted owing to the continous depredation by man.

It was imperative therefore that many of the elite, economically important and endangered species are preserved to make them available when needed.

The conventional methods of storage failed to prevent losses caused due to various reasons.

A new methodology had to be devised for long term preservation of material.

Cryopreservation

- Cryopreservation is a non-lethal storage of biological material at ultra-low temperature. At the temperature of liquid nitrogen (-196 degree) almost all metabolic activities of cells are ceased and the sample can then be preserved in such state for extended peroids.
- However, only few biological materials can be frozen to (-196 degree) without affecting the cell viability.

Germplasm

- A **germplasm** is a collection of **genetic resources** for an organism.
- For plants, the germplasm may be stored as a seed collection (even a large seed bank).
- For trees, in a nursery.
- Animal as well as plant genetics may be stored in a gene bank or cryobank.

History

- Theoreticians of cryopreservation was James Lovelock (born 1919).
- Osmotic stress
- Salt concentration
- Christopher Polge, carried out cryopreservation of first fowl sperm.
- Later, in 1950s they tried it on humans where pregnancy was obtained after insemination of frozen sperm.
- However, the rapid immersion of the samples in liquid nitrogen did not produce the necessary viability to make them usable after thawing.
- Importance of controlled or slow cooling to obtain maximum survival on thawing of the living cells.
- Cryoprotectants were introduced.

Introduction

- In recent years with tremendous increase in population:
- Forest
- Land resources

- Population of medicinal plants
- Aromatic plants species

The conventional methods of germplasm preservation are prone to possible catastrophic losses because of:

1. Attack by pest and pathogens
2. Climate disorder
3. Natural disasters
4. Political and economic causes

The Conservation of germplasm can be done by two methods:

1. *In-situ* preservation: preservation of the germplasm in their natural environment by establishing biospheres, *national* parks etc.
2. *Ex-situ* preservation: in the form of seed or in vitro cultures.

Ex-situ

Advantages

- Small areas can store large amount of material.
- Protection from environmental methods.

Ex-situ

Disadvantages

- Some plants do not produce fertile seeds.
- Loss of seed viability
- Seed destruction by pests, etc.
- Poor germination rate.
- This is only useful for seed propagating plants.
- It's a costly process.

- **Liquid nitrogen** is most widely used material for cryopresevation.
- Dry ice can also be used.

- **Why Liquid nitrogen ?**

✓ Chemically inert

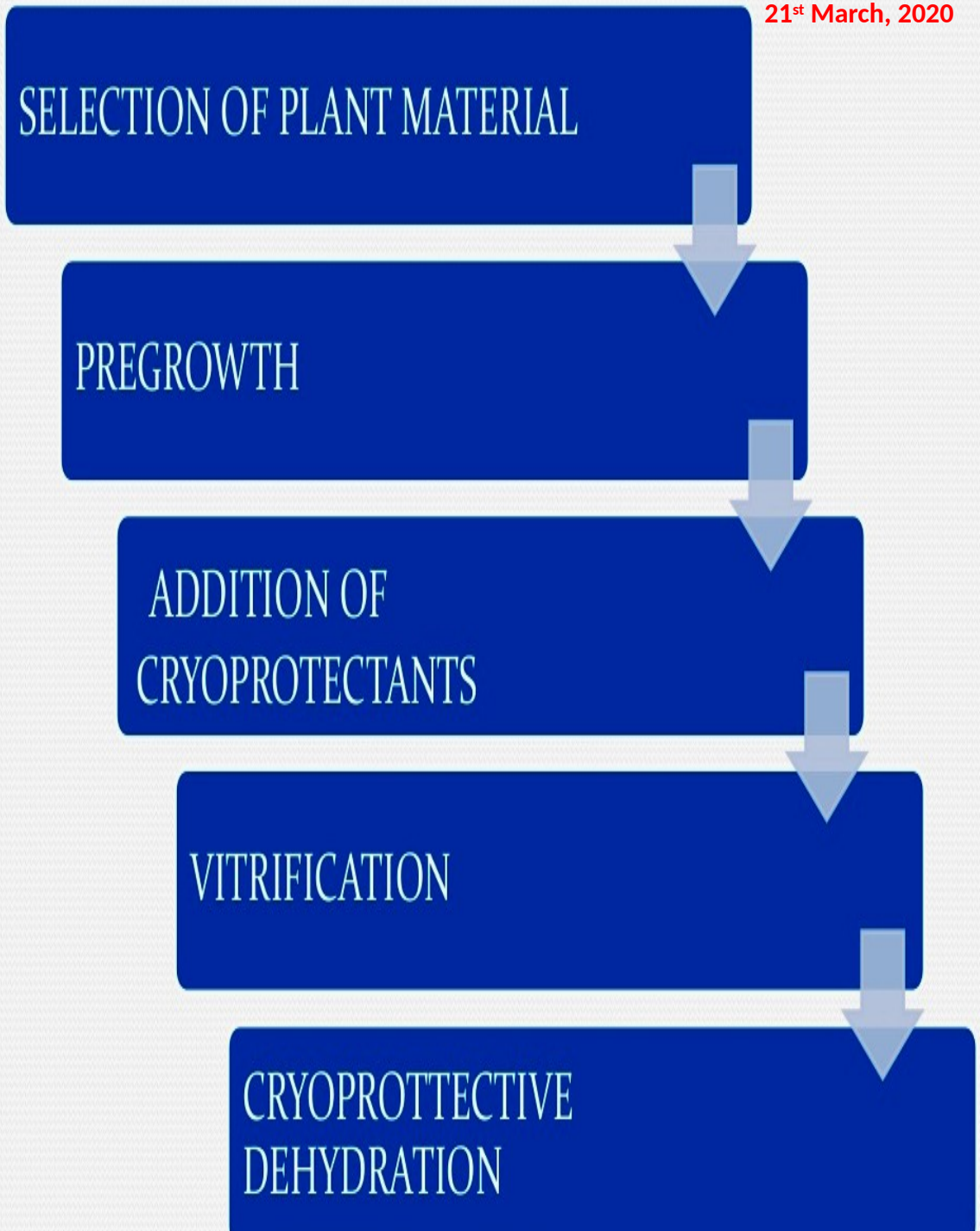
✓ Relatively low cost

✓ Non toxic

✓ Non flammable

✓ Readily available

Steps involved in **Cryopreservation**



ENCAPSULATION AND DEHYDRATION

FREEZING

RAPID FREEZING

SLOW FREEZING

STEPWISE FREEZING

STORAGE

THAWING

DETERMINATION OF SURVIVAL OR VIABILITY

Selection of Plant Material

- Morphological and physiological conditions of plant material influence the ability of explants to survive during cryopreservation.
- Different types of tissues can be used for cryopreservation such as:
- Ovules
- Anther/pollen
- Embryos
- Endosperm
- Protoplast, etc.

FACTORS

- Tissue must be selected from healthy plants.
- Small
- Young
- Rich in cytoplasm
- Meristematic cells can survive better than the larger
- Highly vacuolated cells.

- **Callus** derived from tropical plant is more resistant to freezing damage.
- A rapidly growing stage of callus shortly after 1 or 2 weeks of subculture is best for cryopreservation.
- Old cells at the top of callus and blackened area should be avoided.
- cultured cells are not ideal for freezing. Instead, organized structures such as shoots apices, embryos or young plantlets are preferred.
- Water content of cell or tissue used for cryopreservation should be **low freezable water**, tissues can withstand extremely low temperatures.

Pre-growth

- Pregrowth treatment protect the plant tissues against exposure to liquid nitrogen.
- Pregrowth involves the application of additives known to enhance plant stress tolerance.

E.g.

- ✓ abscisic acid
- ✓ praline
- ✓ trehalose
- ✓ (OTHER EXAMPLES??)

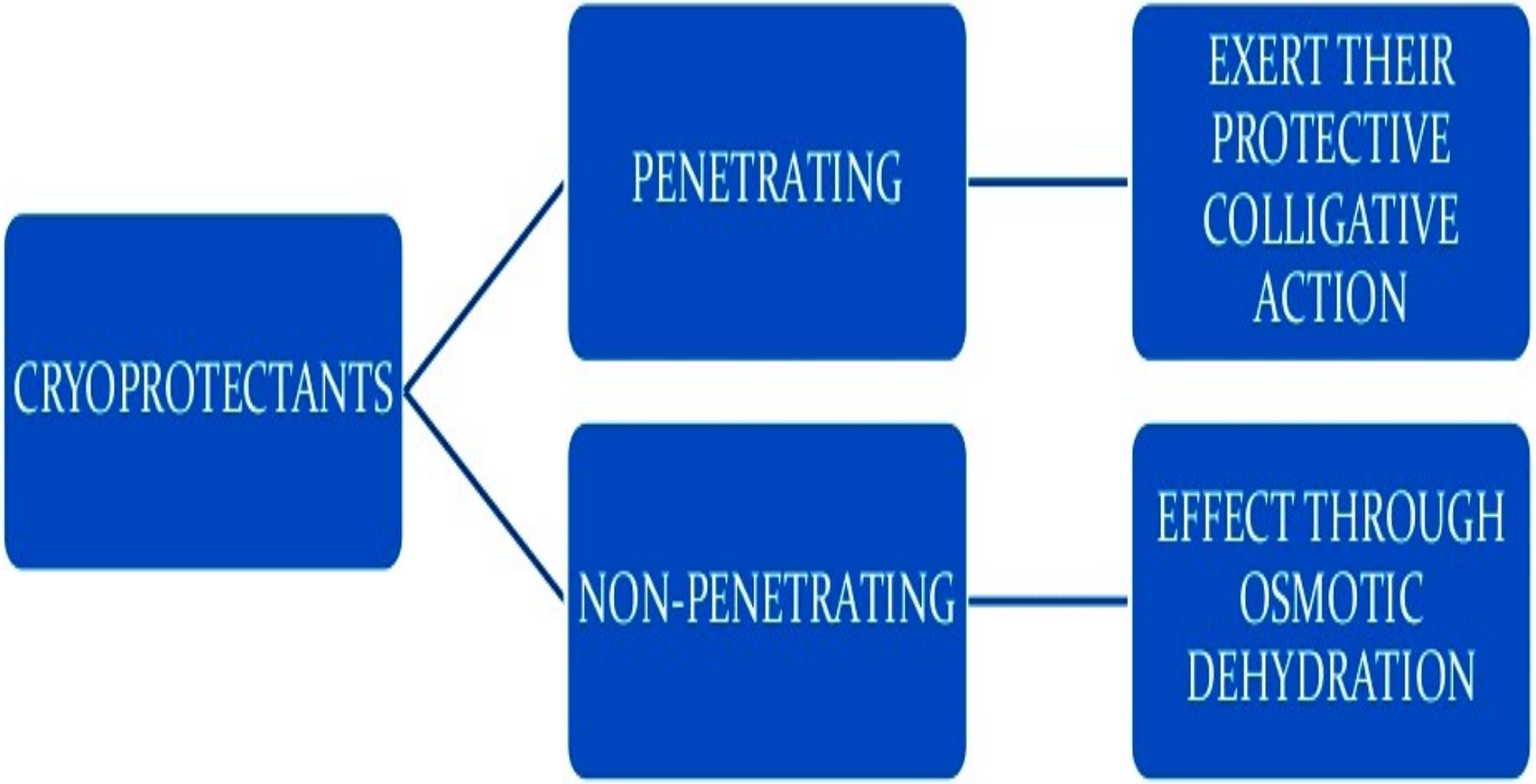
- **Partial tissue dehydration** can be achieved by the application of osmotically active compounds.
- The addition of low concentration of DMSO (1-5%) during pre-growth often improves shoot tip recovery,
- E.g.
- *C. roseus* cells are precultured in medium containing 1M sorbitol before freezing. (Chen et al., 1984)
- *Digitalis* cells were precultured on 6% Mannitol medium for 3 days before freezing. (Seitz et al., 1983)
- *Nicotiana sylvestris* with 6% sorbitol for 2-5 days before freezing. (Maddox et al., 1983)
- (GET MORE EXAMPLES HERE)

Addition of a Cryoprotectant

- A **cryoprotectant** is a substance that is used to protect biological tissue from freezing damage (damage due to ice formation).
- They acts like antifreeze
- They lower freezing temperature
- Increase viscosity and
- Prevents damage to the cells.

- There are **two** potential sources of **cell damage** during cryopreservation.

1. Formation of large ice crystals inside the cell.
2. Intracellular concentration of solutes increase to toxic levels before or during freezing as a result of dehydration.


CRYOPROTECTANTS
PENETRATING
NON-PENETRATING
EXERT THEIR PROTECTIVE COLLIGATIVE ACTION
EFFECT THROUGH OSMOTIC DEHYDRATION

VITRIFICATION

- The term "vitrification" refers to any process resulting in "glass formation", the transformation from a liquid to a solid in the absence of crystallization.
- According to this definition, cells that are properly slow frozen become "vitrified".
- A process where ice formation cannot take place because the aqueous solution is too concentrated to permit ice crystal nucleation. Instead, water solidifies into an amorphous 'glassy' state.

Cryoprotective Dehydration

- **Dehydration** can be achieved by growth in presence of high concentration of osmotically active compounds like
- sugars
- polyols and/or
- In a sterile flow cabinet
- over silica gel.

- **Dehydration** → reduces the amount of water

↓

Ice formation → Increases the osmotic pressure → Depresses its freezing temperature and Promotes vitrification

✓ If cells are sufficiently dehydrated they may be able to withstand immersion in liquid hydrogen.

Encapsulation and Dehydration

- This involves the **encapsulation** of tissues in **calcium alginate beads.**
- Which are pre-grown in liquid culture media containing high concentration of sucrose.
- After these treatments the tissues are able to withstand exposure to liquid nitrogen without application of chemical cryoprotectants.

Cryoprotectants used in cryopreservation

Freezing: Rapid Freezing

- The plant material is placed in vials and plunged into liquid nitrogen and decrease of -300 to -1000°c or more occurs.
- The quicker the freezing is done , the smaller the intracellular ice crystals are.
- Dry ice can also be used in a similar manner.
- This method is technically simple and easy to handle.
- Rapid freezing has been employed for cryopreservation of shoot tips of potato , strawberry , brassica species

Slow Freezing

- Tissue is slowly frozen with decrease in temperature from -0.1 to 10°c/min.
- Slow cooling permits the flow of water from the cells to the outside , thereby promoting extracellular ice formation instead of lethal intracellular freezing.
- This method has been successfully employed for cryopreservation of meristems of peas , potato , cassava , strawberry etc.

- In a normal ice making process, the surface of the cube freezes up much faster than the interior.
- Which "cramps" the interior, clouding it.
- By using very hot (and pure) water inside an insulated environment, you are assuring yourself a very slow freezing that allows the interior to cool down at a rate far closer to that of the exterior, and that lack of "cramping" is what produces such clear ice.

Stepwise Freezing

- In this method slow freezing down to -20 to 40c.
- A stop for a period of approximately 30 min and then additional rapid freezing to -196c is done by plunging in liquid nitrogen.
- Slow freezing permits protective dehydration of the cells and rapid freezing prevents the growing of big ice crystals.
- The Stepwise freezing gives excellent results in strawberry and with suspension cultures.

STORAGE

- Storage of frozen material at correct temperature is as important as freezing.
- The frozen cells/tissues are kept for storage at temperature ranging from -70 to -196°c.
- Temperature should be sufficiently low for long term storage of cells to stop all the metabolic activities and prevent biochemical injury.
- Long term storage is best done at -196°c. ...

Thawing

- It is done by putting ampoule containing the sample in a warm water bath (35 to 40°c).
- Frozen tips of the sample in tubes or ampoules are plunged into the warm water with a vigorous swirling action just to the point of ice disappearance.
- It is important for the survival of the tissue that the tubes should not be left in the warm water bath after ice melts .
- just a point of thawing quickly transfer the tubes to a water bath maintained at room temperature and continue the swirling action for 15 sec to cool the warm walls of the tube.
- Tissue which has been frozen by encapsulation/dehydration is frequently thawed at ambient temperature.

Determination of Survival/Viability

- Regrowth of the plants from stored tissues or cells is the only test of survival of plant materials.
- Various viability tests include Fluorescien diacetate (FDA) staining , growth measurement by cell number , dry and fresh weight.
- Important staining methods are:

❑ Triphenyl Tetrazolium Chloride (TTC)

❑ Evan's blue staining.

Triphenyl Tetrazolium Chloride (TTC) Assay

- Cell survival is measured by amount of red formazan product formed due to reduction of TTC assay which is measured spectrometrically.
- Only the viable cells which contain the enzyme mitochondrial dehydrogenase which reduces TTC to red formazan will be stained and dead cells will not take up the dye.

Evan's Blue Staining

- One drop of 0.1% solution of Evan's blue is added to cell suspension on a microscope slide and observed under light microscope.
- Only non viable cells (dead cells) stain with Evan's blue. % of viable cells = Number of fluorescent cells x 100 total no of cells(viable + non-viable).
- Individual cell viability assayed with Evan's blue dye and fluorescein diacetate.

Measurement of growth of Cell Cultures

- Fresh and dry weight measurements
- Increase in cell number
- Packed cell volume (PCV)
- Molecular protien and DNA
- Mitotic index
- Medium component calibration
- Conductivity of medium
- Cellular protien

Fresh & Dry Weight Measurement

For callus

- Transfer the entire callus to pre-weighed weighing boat.
- Determine the fresh weight.

For cell suspension culture

- Collect the cells on a pre weighed nylon membrane
- Determine the fresh weight

Alternative Method

- Weigh an empty centrifuged tube.
- Add cell suspension culture to the tube.
- Centrifuged the tube for 5-10 minutes.
- Check the supernatant fluid, it should not show any cells.
- Later without disturbing the pellet separate the supernatant solution with the help of pipette.
- Weigh the tube along with the cells to determine the fresh weight.

- Dry the samples in an oven for 60 degree untill no change in the dry weight is measured.

- Growth index (GI) = $\frac{\text{Final weight of callus or cells}}{\text{Initial weight of the callus or cells}}$

Increase in Cell Number

- Haemocytometer can be used
- Callus or cells suspension is needed to be macerated
- Maceration fluid is an equal volume of 10% chromic acid and 10% nitric acid.
- Treat this mix foe 5 to 10 minutes at 60 degree.
- After cooling, shake the container vigorously to loosen the cell clumps.
- 1% macerozyme incubate flask overnight under growth condition.

Packed Cell Volume

- Suitable for suspension culture only.
- Simple and can be determined at any stage of growth.
- A small volume(10ml) is aseptically sampled and placed in graduated conical centrifuged tube.
- Total volume of a cell pellet is determined after centrifuging at a constant speed of (500g) for specific time (5 mins).
- PCV is typically expressed as a percentage of the total volume in tube.

Molecular Protein & DNA

- The components like DNA and protien are usually determined by using well established techniquese.g. Barford's reagent for protien.

Mitotic Index

- Mitotic index is an estimate of the number of cells of population in the stages like prophase., metaphase, anaphase and telophase.
- It is simple but time cnsuming.
- Mitotic index:
- (Number of nuclei in mitosis/ total number of nuclei scored) x 100

Cryopreservation Instruments

- A reliable source of liquid nitrogen
- Safety equipment (gloves, apron, face shield, pumps for dispensing liquid nitrogen from a large storage dewars, trolleys for the transport of dewars).
- Small (1-2 litre) liquid nitrogen resistant dewar(s)
- Dewar(s) for the routine storage of liquid nitrogen.
- Dewar(s) for the long term storage of spicemens
- Cryovilas, straws, boxes, canes, racks.
- A refrigerent (-20 degree)
- A programmable feezer wit dewar and pump
- A water bath for thawing at 40 to 50 degree.

Application of Cryopreservation:

Conservation of Genetic Material

- Cryopreservation provides an opportunity for conservation of endangered medicinal plants.
- Cryopreservation has been used successfully to store a range of tissue types , including meristems , anthers/pollens and embryos.

Freeze Storage of Cell Culture

- A cell line to be maintained has to be subcultured and transferred periodically and repeatedly over an extended period of time.
- cryopreservation is an ideal approach to suppress cell division to avoid the need for periodical subculturing

Maintenance of Disease free Stock

- Pathogen free stocks of rare plant material could be frozen and propagated when needed.
- Cold acclimatization and frost resistance
- A cryopreserved tissue culture would provide a suitable material for selection of cold resistant mutant cell lines , which could later differentiate into frost resistance plants.

Gene Bank

- Gene banks are a type of biorepository which preserve genetic material.
- In plants, this could be by freezing cuts from the plant, or stocking the seeds.
- In animals, this is the freezing of sperm and eggs in zoological freezers until further need..
- In an effort to conserve agricultural biodiversity, gene banks are used to store and conserve the plant genetic resources of major crop plants and their crop wild relatives.
- There are many gene banks all over the world, with the Svalbard Global Seed Vault being probably the most famous one.

- *Gene Banks are a type of biorepository which preserve genetic material.*
- *A collection of seed plants, tissue cultures etc. from potentially useful species , especially species containing genes of significance to the breeding of crops.*
- *In plants this could be done by freezing cuts from the plants or stocking the seeds.*
- With corals, fragments are taken which are stored in water tanks under controlled conditions.
- Plant genetic material in a 'gene bank' is preserved at -196° Celsius in Liquid Nitrogen as mature seed (dry) or tissue (meristems).
- *In Plants it is possible to unfreeze the material and propagate it, however In animals a living female is required for artificial insemination.*
- *In animals this the freezing of sperm and eggs in zoological freezers. It is difficult to utilize frozen animals sperms and eggs,* there are many examples of it being done successfully.
- The database of the largest gene banks in the world can be queried via a common website, Genesys.

- Gene banks exist to conserve the genetic diversity of wild and domesticated organisms that humans depend on for food, fiber, medicine & energy.
- Over 7.2 million plant germplasm accessions are housed in ~1,750 national and international gene banks.
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- There are many gene banks all over the world, with the Svalbard Global Seed Vault being probably the most famous one.
- Accession is the common term given to an individual sample in a gene bank, such as a distinct species or variety.
- Most accessions are poorly characterized; few are ever used.
- Gene banks manage both genetic resources and information about those resources.
- Diverse genetic resources are essential to improving the productivity, nutritional quality and sustainability of agricultural systems.
- Rapid scientific/ technological developments have changed the way scientists explore and understand natural variation.
- Gene bank managers must stay abreast of scientific developments to fulfill their responsibilities to the public.

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Svalbard Global Seed Vault in Norway, 2008

CIMMYT, 1966

Cryopreservation, NBPGR, 1997

Herbal garden

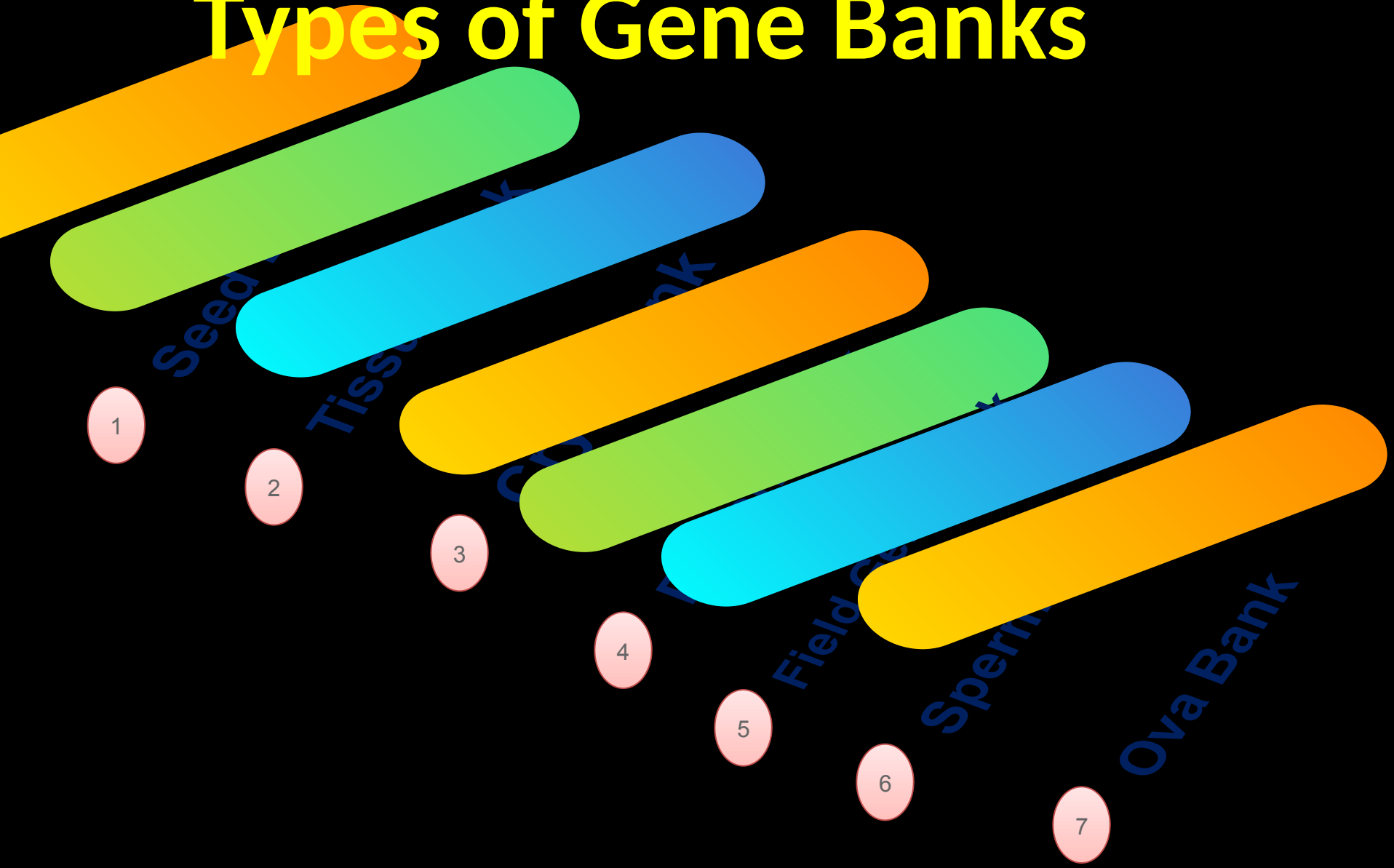
Natural Habitat

Field gene bank, JNKVV

HISTORY

- Sir Otto Frankel coined the term Genetic resources in 1968 to aware the plant breeders of this gradual loss of germplasm.
- 1970 In USA initiating action to collect, conserve, evaluate and utilize the plant germplasm resources ,when southern corn leaf blight was out broke.
- Alphonse de Candolle was the first botanist to attempt to locate the origin of crop plants, in 1882 he published a *book "Origin Of Cultivated plants."*
- 1926 Nikolai Ivanovich Vivelov, Russian explorer, geneticist, agronomist organized world wide exploration for collecting the seeds and propagating material of large number of cultivated crops, wild and related species.
- 1951 Vavilov proposed eight centre of origin and three sub centre of different plant species.
- 1961 FAO organized the first International technical meeting on plant exploration & introduction.
- 1968 The Crop Ecology & Genetic Resources Unit (CEGRU) of FAO was established.
- 1974 International Board Of Plant Genetic Resources (IBPGR) was established in Rome.
- 1992 IBPGR was transformed to a new autonomous organization International Plant Genetic Resources Institute (IPGRI) to assist the countries (developing).
- 1905 conservation of PGRs was initiated in India.
- 1976 NBPGR was established in New Delhi for conservation of various crop species.
- 1985 NBAGR was established in Karnal for conservation of various Animal species.

Types of Gene Banks



Seed Bank

- The seed bank preserves dried seeds by storing the
low temperature.
- Spores and pteridophytes are conserved in seed banks but other seedless plants such as tuber crops cannot be preserved this way.
- The largest seed bank in the world is the Millennium seed bank housed at the Welcome Trust Millennium Building (WTMB) in London.

Tissue Bank

- In this technique buds, protocorms and meristematic cells are conserved through particular light and temperature arrangements in a nutrient media.
- This is used to preserve seedless plants and plants which reproduce asexually.

Cryo Bank

- In this technique a seed or embryo is preserved at a very low temperature.
- It is usually preserved in liquid nitrogen at - 196 degrees.
- This is helpful for the conservation of species facing extinction.

Pollen Bank

- This is a method in which pollen grains are store
- We can make plants which are facing extinction in the present world using this technique .
- By this technique we can make plants with one set chromosomes.

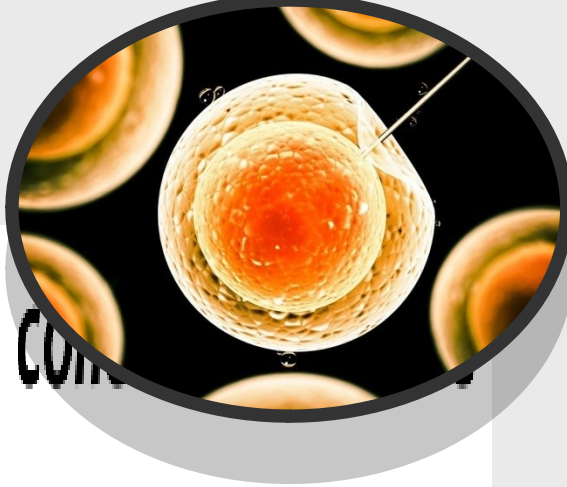
Field Gene Bank

- This is a method of planting plants for the conservation of genes.
- For this purpose we construct ecosystem artificially .
- Through this method one can compare the difference among plants of different species in detail.
- It needs more land, adequate soil, weather etc.
- Germplasm of important crops are conserved through this method.
- 42,000 varieties of rice are conserved in the Central Rice Research Institute in Orissa.

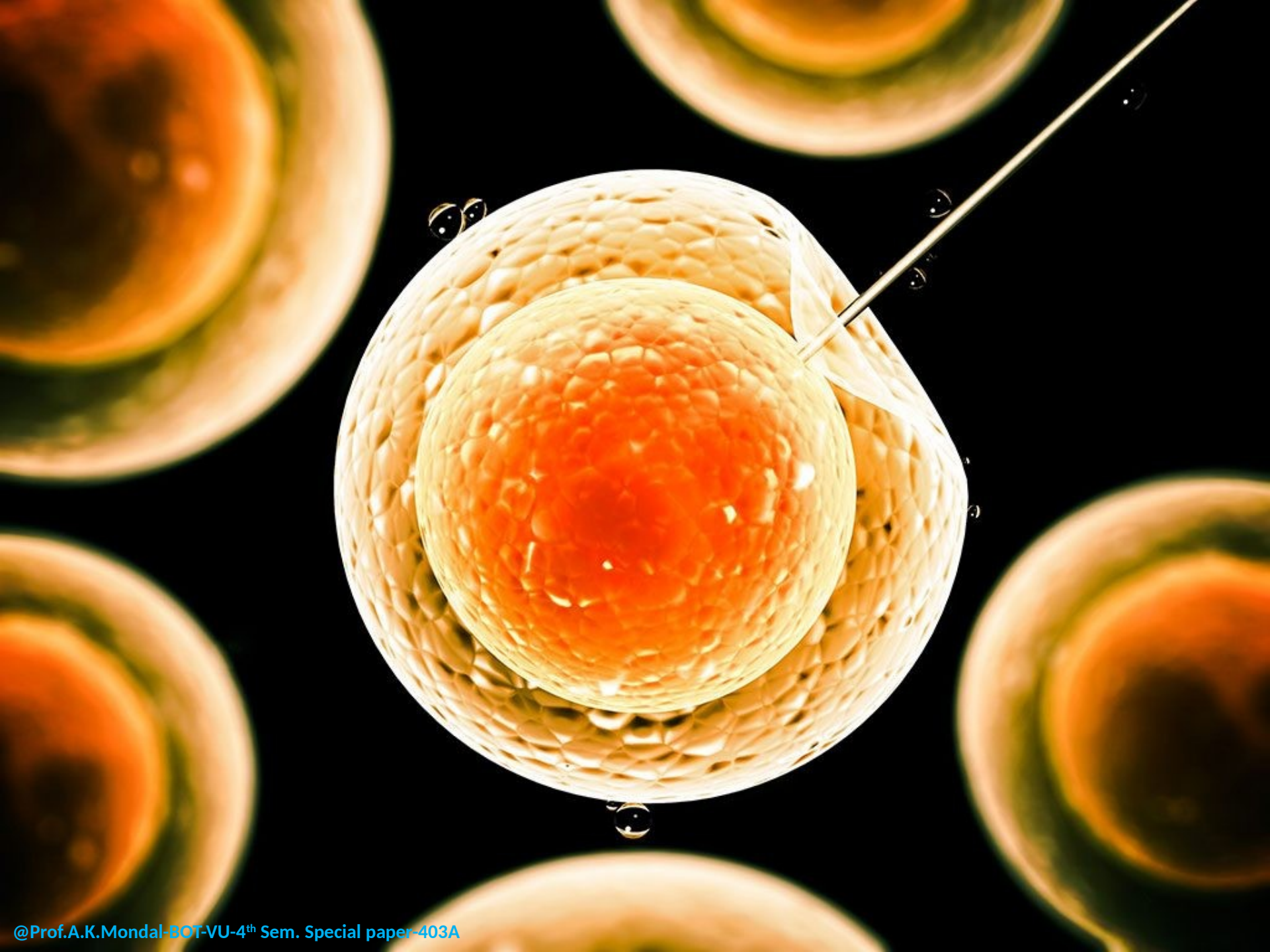
Sperm Bank

- A sperm bank, semen bank is a facility or enterprise tha and store human sperm donors for use by women who ,for whatever reason , need donor provided sperm to achieve pregnancy.
- Sperm donated by the sperm donor is known as donor sperm.
- And the process for introducing sperm into women is called Artificial insemination.

Ova Bank



- Ova bank or egg cell bank is a facility that co… human ova primarily from the ova donors.
- The purpose of achieving pregnancy of donor (i.e. to overcome issues of fertility) or through third party reproduction .
- Ova donated in this way from the donor is known as donor ova.



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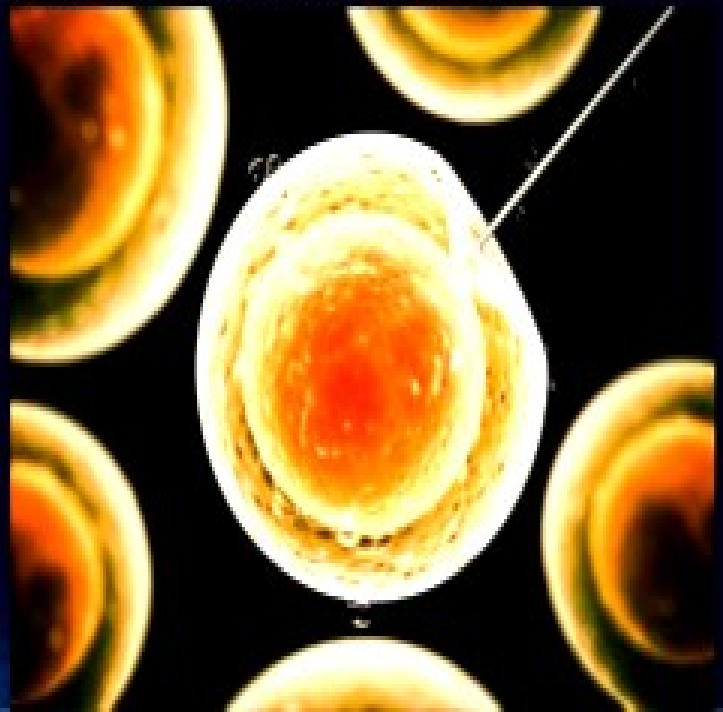


Botanic Garden

- Botanic gardens - over 2,500, in 148 countries, with about 6.13 million accessions

Purpose of Gene Bank

- Physical facilities for maintaining collections of live plant materials – domesticated cultivated plants – wild plant species (crop wild relatives and other wild plant species useful for food and agriculture and other end uses) – entire plants, seeds, pollen, embryos, meristems, cells, or DNA, depending on the biology of the species
- *It is a facility for maintaining crop diversity .*
- *Usually this diversity is in the form of seeds, stored and conserved in a frozen state .*
- *Some gene banks use normal household freezers for this purpose.*
- *The ideal temperature is between -10and -20.*
- *Each different type is stored in its own container.*
- *Such as a bottle, a can or a sealed aluminum foil package.*

- Maintaining material in genebanks is often termed 'ex situ conservation' – defined as 'the conservation of components of biodiversity outside their natural habitats' (CBD, 1992).
- Whereas in situ conservation is maintenance of viable population in their natural surroundings – a dynamic system which allows the biological resources to evolve and change over time through natural selection processes.
- Both concepts are therefore fundamentally different but are complementary.

Gene Bank therefore are managed so as to:

- Maintain the genetic integrity of its accessions
- Make the accessions easily available to users of germplasm
- Provide the raw material for plant breeding and basic biological research – Accessions of crop wild relatives are particularly valuable as sources of gene providers
- Provide germplasm for restoration of lost crops after natural or man-made catastrophes

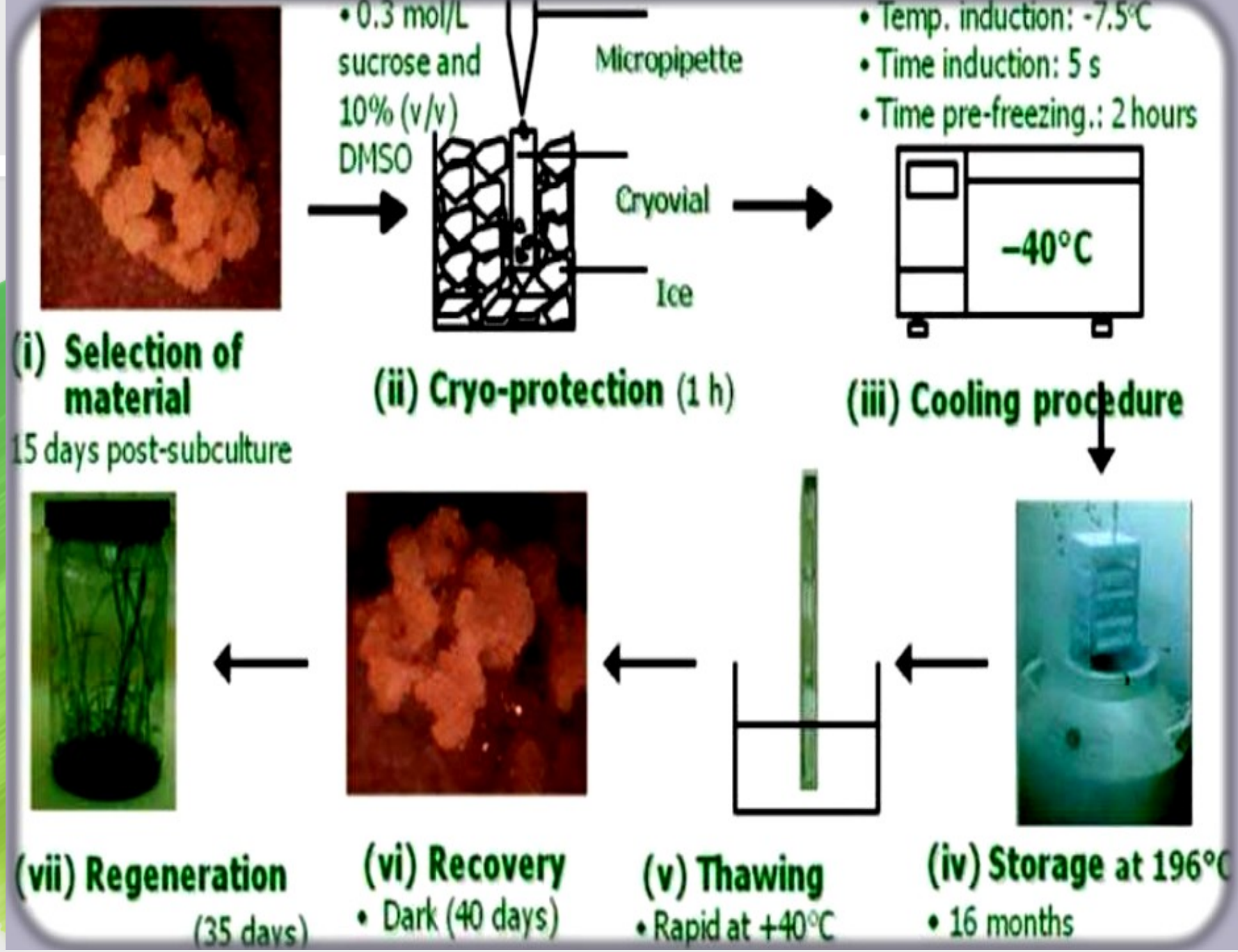
Gene Bank for Various Crops in India

SL. NO.	CROP	CENTER
1	Wheat	DWR, Karnal
2	Rice	CRRI,Cuttack,IGKV,Raipur
3	Potato	CPRI,Shimla
4	Cotton	CICR,Nagpur
5	Sugarcane	SBI,Coimbatore
6	Tobacco	CTRI, Rajahmundry
7	Pulses	IIPR, Kanpur
8	Forage crops	IGFRI,Jhansi
9	Tuber crops(except potato)	CTCRI,Trivendaram ,Kerala
10	Plantation crops	CPCRI,Kasargod
11	Oilseeds crop	DOR Hyderabad
12	Horticultural crops	IIHR, Bangalore
13	Sorghum	NRC Sorghum ,Hyderabad
14	Soybean	NRC Soybean ,Indore
15	Groundnut	NRC Groundnut, Junagarh
16	Maize	IARI, New Delhi

Activities in Gene Bank

The main activities in the development and management of a gene bank include:

- **Collecting and Acquisition** – assembling the collection
- **Processing** – assessing the quantity, viability, health of samples and preparation for storage
- **Storage** – in a cold store, laboratory or in the field
- **Regeneration and Multiplication** – periodically rejuvenating and increasing the material
- **Characterization and Evaluation**
- **Documentation, Inventory** – maintaining and making available detailed records on each sample
- **Distribution** – of clean, disease-free seeds, or other planting material, to requestors


• 0.3 mol/L sucrose and 10% (v/v) DMSO
Micropipette
Cryovial
Ice
• Temp. induction: -7.5°C
• Time induction: 5 s
• Time pre-freezing.: 2 hours
–40°C
(i) Selection of material
15 days post-subculture
(ii) Cryo-protection (1 h)
(iii) Cooling procedure
(iv) Storage at 196°C
• 16 months
(v) Thawing
• Rapid at +40°C
(vi) Recovery
• Dark (40 days)
(vii) Regeneration
(35 days)

- **National Seed Storage Laboratory** (NSSL) (Fort Collins, Colorado, USA): 2,100 accessions of apple (dormant buds) .
- National Clonal Germplasm Repository (NCGR) of Corvallis (USA): 104 accessions of pear (shoot tips).
- International Potato Centre (CIP) (Lima, Peru) : 345 potato accessions.
- Tissue Culture BC Research Inc .(Vancouver, BC, Canada) : 5000 accessions representing 14 conifer species.

- **AFOCEL** (Association Foret Cellulose) of France, with over 100 accessions of elm (dormant buds).
- **National Institute of Agro biological Resources** (NIAR) of Japan, with about 50 accessions of mulberry.
- **IRD** (Montpellier, France) : 80 accessions of oil palm .
- **German Collection of Micro-organisms and Cell Cultures** (DSMZ) (Braunschweig, Germany) : 519 old potato varieties.
- **INIBAP**, Laboratory of Tropical Crop Improvement, K.U.Leuven (Heverlee, Belgium) : 440 banana accessions.


ThanK
You
Any Questions??????