Cryobiology

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Definition

- Cryobiology is actually the study of living systems at any temperature below the standard physiological range.
- Usually thought of as the study of the effects of subfreezing temperatures on biological systems
- Stands at the interface between physics and biology.
- Plant, insect, and vertebrate natural cold hardiness and sensitivity; freeze-drying; supercooling; cryosurgery; frostbite; and deliberate cryopreservation

Cold hardiness and Sensitivity in nature

- Tree twigs can survive direct immersion in liquid nitrogen after suitable pre-conditioning.
- It turns out they achieve this by manufacturing proteins and sugars that allow the cytoplasm to turn into a glass at temperatures about 30 to 40 degrees below zero;
- Once the plant cells vitrify, they are immune to most low-temperature excursions. Certain lichens are even more dazzling, vitrifying in toto upon cooling and warming, without previous crystallization

Cold hardiness and Sensitivity in nature

- Certain plants achieve the same control of ice by elaborating a physical barrier between sensitive areas (the apical meristems) and the loci of ice formation, such that water can leave the meristems and deposit on ice in the ice-tolerant area, but ice cannot grow through the barrier to invade and thereby to kill the meristem.
- The meristem can survive the dehydration, and so it survives the winter.

Freeze drying

- Reported to yield viable cells. Works best for bacteria and other prokaryotes.
- The LifeCell Corporation invented a technique by which cells are "vitri-dried": the cells are cooled so rapidly that ice either does not form or forms such small crystals that they do not damage the cells, after which space-quality vacuums are drawn to distill off the water at very low temperatures

Supercooling

- Supercooling has recently formed the basis of a British company, Pafra, Ltd., which preserves enzymes and even whole cells by cooling them in tiny droplets of water to temperatures several degrees below their freezing point.
- Because the probability of spontaneous freezing is small in such small volumes, and because the droplets are prevented from touching each other by being dispersed in a non-aqueous phase as an emulsion, stable supercooling of great magnitude (e.g., -10 to -20 degrees C) can be attained for months.

Supercooling

- Supercooling can cause enzyme denaturation, since cooling weakens the hydrophobic interactions that give rise to protein folding and membrane self-assembly, but such denaturation is usually readily reversed spontaneously when the proteins are warmed up.

Cryosurgery

- Cryosurgery works by exposing cells in the patient to very rapid cooling to deep subzero temperatures.
- Rapid cooling, for basic physical reasons, causes water inside cells to freeze, whereas the slow cooling found in nature and usually used for cryopreservation causes intracellular water to leave cells and freeze extracellularly.
- Intracellular freezing tends to be lethal, and its lethality is enhanced by slow warming, which allows intracellular ice to rearrange itself into a simpler structure, in the process literally grinding up the cellular interior.

Cryopreservation (preservation in liquid nitrogen, -196°C) of plant germplasm

- The term ‘cryopreservation’ (cryogenic preservation) refers to the storage of cells, tissues and organs at the ultra-low temperature of liquid nitrogen (-196°C).
- At this temperature, the vegetative cells enters in a state of “absolute quiescence”, as all the physical and biochemical reactions are practically halted; in this particular condition, conservation time becomes unlimited.

Cryopreservation (preservation in liquid nitrogen, -196°C) of plant germplasm

- Application of cryogenics to the conservation of plant material, proposed for the first time in the year 1968 for the maintenance of cell cultures, is today a reality for the conservation of differentiated organs and tissues as well.
- When the cells are led to this extreme ultra-cold condition following appropriate "preparatory" procedures, their viability is preserved and, when brought back to standard culture conditions, they can recover full functionality.

Cryopreservation (preservation in liquid nitrogen, -196°C) of plant germplasm

- possibility to put in conservation a wide range of plant organs and tissues (shoot tips from in vitro culture, seeds and embryonic axes, somatic embryos, bulbils, buds collected in the field, pollens),
- the limited space necessary for the conservation (a medium-size dewar for liquid nitrogen contains 5,000-10,000 explants),

Cryopreservation (preservation in liquid nitrogen, -196°C) of plant germplasm

- the low conservation cost (practically, the cost necessary for the cryobank control and for the provision of liquid nitrogen, a liquid gas cheap and easy to find), the maintenance of specimens in absolute genetic and sanitary security.
- possible to preserve cell and hairy root cultures producing secondary metabolites of industrial and pharmacological interest, as well as callus cultures, embryogenic and/or genetically transformed.

Cryopreservation (preservation in liquid nitrogen, -196°C) of plant germplasm

- Recently, the cryogenic tecnology showed its effectiveness also for the recovery of virus-, phytoplasm- and bacterium-free plants (cryotherapy).
- Trees and Timber Institute, coordinated by Dr. Maurizio Lambardi, studies the application of the cryogenic technology to the conservation of woody plant germplasm (fruits, timber and ornamental species).
- Effective procedures have been developed for the cryopreservation of shoot tips from white poplar, pear, plum, kaki and olive, seeds of Citrus and Pistacia spp., embryogenic callus of olive, horse chestnut and ash.

Cryopreservation (preservation in liquid nitrogen, -196°C) of plant germplasm

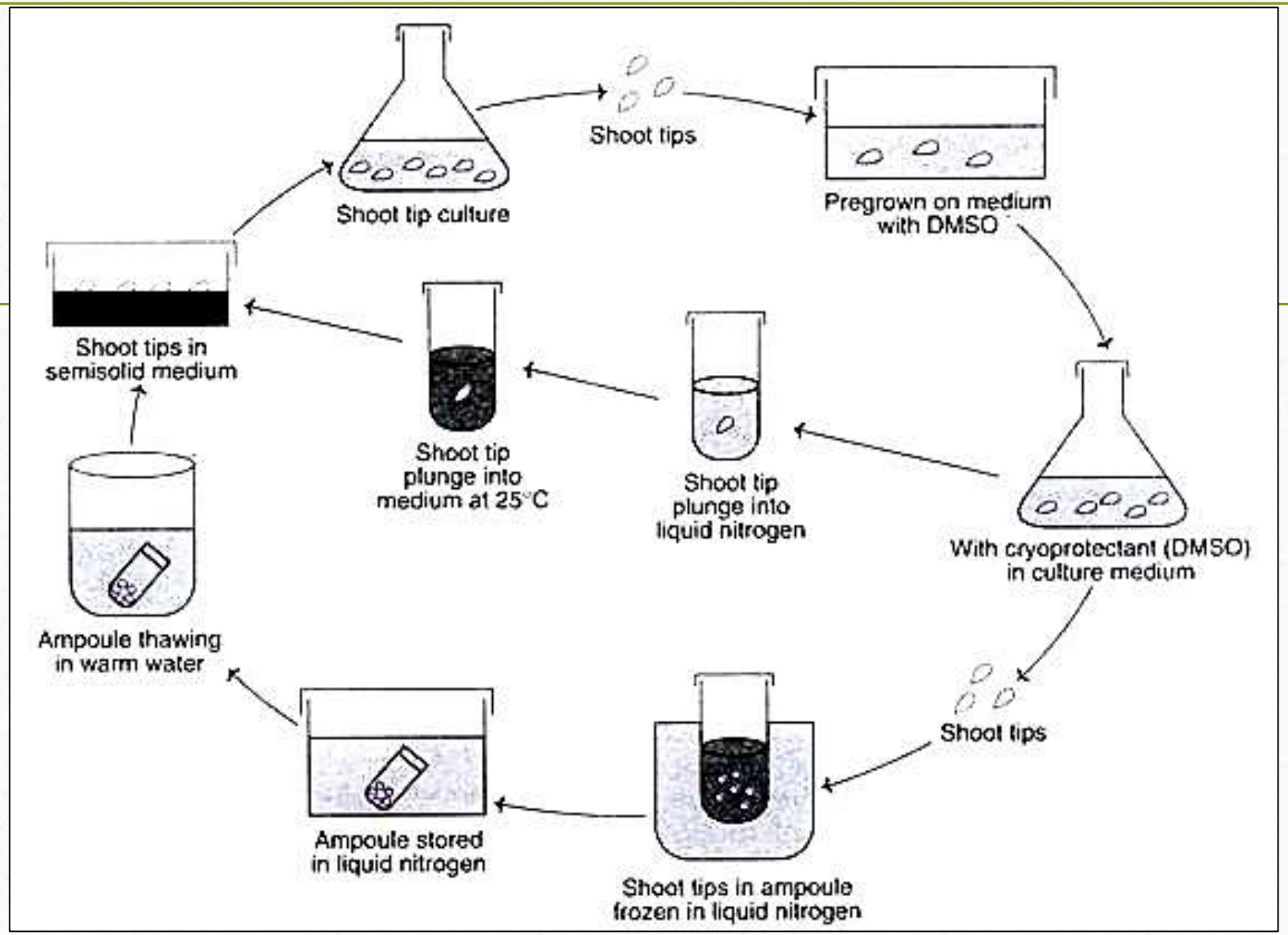
- The vitrification solution PVS2 is largely used with both naked and encapsulated explants (synthetic seeds).
- An innovative procedure, based on the cryopreservation of dormant buds which, after thawing, are used for chip-budding onto rootstocks, has been recently applied to the conservation of ancient apple germplasm.
- Important information on the micro-morphological characteristics of explants recovered from cryopreservation are obtained by means of histo-anatomical observations.

Cryopreservation methods

- i. Over solid carbon dioxide (at -79°C)
- ii. Low temperature deep freezers (at -80°C)
- iii. In vapour phase nitrogen (at -150°C)
- iv. In liquid nitrogen (at -196°C)

Cryopreservation mechanism

- The technique of freeze preservation is based on the transfer of water present in the cells from a liquid to a solid state.
- Due to the presence of salts and organic molecules in the cells, the cell water requires much more lower temperature to freeze (even up to -68°C) compared to the freezing point of pure water (around 0°C).
- When stored at low temperature, the metabolic processes and biological deteriorations in the cells/tissues almost come to a standstill.


Shoot tip culture
Shoot tips
Pregrown on medium with DMSO
With cryoprotectant (DMSO) in culture medium
Shoot tip plunge into liquid nitrogen
Shoot tip plunge into medium at 25°C
Shoot tips in semisolid medium
Ampoule thawing in warm water
Shoot tips
Shoot tips in ampoule frozen in liquid nitrogen
Ampoule stored in liquid nitrogen

Cryopreservation protocol

- 1. Development of sterile tissue cultures
- 2. Addition of cryoprotectants and pretreatment
- 3. Freezing
- 4. Storage
- 5. Thawing
- 6. Re-culture
- 7. Measurement of survival/viability
- 8. Plant regeneration.

Development of sterile tissue culture

- The selection of plant species and the tissues with particular reference to the morphological and physiological characters largely influence the ability of the explant to survive in cryopreservation.
- Any tissue from a plant can be used for cryopreservation e.g. meristems, embryos, endosperms, ovules, seeds, cultured plant cells, protoplasts, calluses.
- Among these, meristematic cells and suspension cell cultures, in the late lag phase or log phase are most suitable.

Addition of cryoprotectants and pretreatment

- Cryoprotectants are the compounds that can prevent the damage caused to cells by freezing or thawing.
- The freezing point and super-cooling point of water are reduced by the presence of cryoprotectants.
- As a result, the ice crystal formation is retarded during the process of cryopreservation.

Addition of cryoprotectants and pretreatment

- There are several cryoprotectants which include dimethyl sulfoxide (DMSO), glycerol, ethylene, propylene, sucrose, mannose, glucose, proline and acetamide.
- Among these, DMSO, sucrose and glycerol are most widely used. Generally, a mixture of cryoprotectants instead of a single one is used for more effective cryopreservation without damage to cells/tissues.

Slow-freezing method

- The tissue or the requisite plant material is slowly frozen at a slow cooling rates of 0.5-5°C/min from 0°C to -100°C, and then transferred to liquid nitrogen.
- The advantage of slow-freezing method is that some amount of water flows from the cells to the outside.

Slow-freezing method

- This promotes extracellular ice formation rather than intracellular freezing.
- As a result of this, the plant cells are partially dehydrated and survive better.
- The slow-freezing procedure is successfully used for the cryopreservation of suspension cultures.

Rapid freezing method

- This technique is quite simple and involves plunging of the vial containing plant material into liquid nitrogen.
- During rapid freezing, a decrease in temperature -300° to -1000°C/min occurs.
- The freezing process is carried out so quickly that small ice crystals are formed within the cells.

Rapid freezing method

- Further, the growth of intracellular ice crystals is also minimal.
- Rapid freezing technique is used for the cryopreservation of shoot tips and somatic embryos.

Stepwise freezing method

- This is a combination of slow and rapid freezing procedures (with the advantages of both), and is carried out in a stepwise manner.
- The plant material is first cooled to an intermediate temperature and maintained there for about 30 minutes and then rapidly cooled by plunging it into liquid nitrogen.
- Stepwise freezing method has been successfully used for cryopreservation of suspension cultures, shoot apices and buds.

Dry freezing method

- Some workers have reported that the non-germinated dry seeds can survive freezing at very low temperature in contrast to water-imbibing seeds which are susceptible to cryogenic injuries.
- In a similar fashion, dehydrated cells are found to have a better survival rate after cryopreservation.

Storage

- Maintenance of the frozen cultures at the specific temperature is as important as freezing.
- In general, the frozen cells/tissues are kept for storage at temperatures in the range of -70 to -196°C.
- However, with temperatures above -130°C, ice crystal growth may occur inside the cells which reduces viability of cells.
- Storage is ideally done in liquid nitrogen refrigerator — at 150°C in the vapour phase, or at -196°C in the liquid phase.

Storage

- The ultimate objective of storage is to stop all the cellular metabolic activities and maintain their viability.
- For long term storage, temperature at -196°C in liquid nitrogen is ideal. A regular and constant supply of liquid nitrogen to the liquid nitrogen refrigerator is essential.
- It is necessary to check the viability of the germplasm periodically in some samples. Proper documentation of the germplasm storage has to be done.

Storage

- i. Taxonomic classification of the material
- ii. History of culture
- iii. Morphogenic potential
- iv. Genetic manipulations done
- v. Somaclonal variations
- vi. Culture medium
- vii. Growth kinetics

Thawing

- Thawing is usually carried out by plunging the frozen samples in ampoules into a warm water (temperature 37-45°C) bath with vigorous swirling.
- By this approach, rapid thawing (at the rate of 500- 750°C min-1) occurs, and this protects the cells from the damaging effects ice crystal formation.

Thawing

- As the thawing occurs (ice completely melts) the ampoules are quickly transferred to a water bath at temperature 20-25°C.
- This transfer is necessary since the cells get damaged if left for long in warm (37-45°C) water bath.
- For the cryopreserved material (cells/tissues) where the water content has been reduced to an optimal level before freezing, the process of thowing becomes less critical.

Re-culture

- In general, thawed germplasm is washed several times to remove cryoprotectants.
- This material is then re-cultured in a fresh medium following standard procedures.
- Some workers prefer to directly culture the thawed material without washing. This is because certain vital substances, released from the cells during freezing, are believed to promote in vitro cultures.

Measurement of survival/viability

- The viability/survival of the frozen cells can be measured at any stage of cryopreservation or after thawing or re-culture.
- The techniques employed to determine viability of cryopreserved cells are the same as used for cell cultures.
- Staining techniques using triphenyl tetrazolium chloride (TTC), Evan's blue and fluorescein diacetate (FDA) are commonly used.

Measurement of survival/viability

- The best indicator to measure the viability of cryopreserved cells is their entry into cell division and regrowth in culture.
- This can be evaluated by the following expression.

$$\frac{No.of\ cells/organs\ growing}{No.of\ cells/organs\ thawed} \times 100$$

Plant regeneration

- The ultimate purpose of cryopreservation of germplasm is to regenerate the desired plant.
- For appropriate plant growth and regeneration, the cryopreserved cells/tissues have to be carefully nursed, and grown.
- Addition of certain growth promoting substances, besides maintenance of appropriate environmental conditions is often necessary for successful plant regeneration.

Precautions

- i. Formation ice crystals inside the cells should be prevented as they cause injury to the organelles and the cell.
- ii. High intracellular concentration of solutes may also damage cells.
- iii. Sometimes, certain solutes from the cell may leak out during freezing.
- iv. Cryoprotectants also affect the viability of cells.
- v. The physiological status of the plant material is also important.

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ENDANGERED PLANT *signature*

Returning to the Wild

Species: Autumn buttercup (*Ranunculus aestivalis*)

Status: Endangered, U.S.

Location: Nature Conservancy, Sevier Valley Preserve, Panguitch, Utah

CREW'S CRYOBIOBANK®

mission is the CryoBioBank. CREW's CryoBioBank was created to provide a safe haven for some of the world's most valuable and irreplaceable biological samples from rare and endangered plants and animals. However, the CryoBioBank's reservoir of genetic diversity is not a static collection. Instead, samples are added to

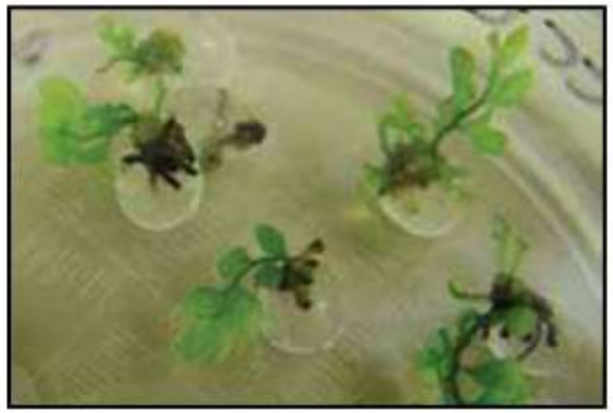
CREW's Frozen Garden

- The collection of plant samples within the CryoBioBank is called the Frozen Garden.
- There are over 125 different plant species represented in the Frozen Garden.
- Samples include shoot tips, pollen, seeds and spores.

History and Success stories

1986-1990

Dr. Valerie Pence establishes the Plant Research Division at CREW and initiates the Endangered Plant Propagation Program.



1996-2000

CREW pioneers "in vitro collecting" technique, and demonstrates its effectiveness in collecting plant tissues from Trinidad, Costa Rica, and several habitats in the United States.

2001-2005

Successful propagation of CREW's 20th rare plant species.

Tree embryos shown to be viable after 10 years in CREW's CryoBioBank.

2006-2011

Pollen, spores, and shoot tips in CREW's CryoBioBank prove viable after 10-15 years in storage.

Success stories

Black Walnut Embryos Revive After 23 Years on Ice

http://blog.cincinnatizoo.org/2017/01/30/black-walnut-embryos-revive-after-23-years-on-ice/

THE NEW INDIAN EXPRESS
Tuesday, February, 13, 2018

Freeze! To save plants' genes for posterity, TNAU building cryobank

By Sushmitha Ramakrishnan | Express News Service | Published: 09th October 2017 02:33 AM | Last Updated: 09th October 2017 07:35 AM | A+ A A-

Representational image

CHENNAI: Soon genes from plants of the State can be stored for over 1,000 years. Tamil Nadu Agricultural University is building a cryobank — a gene bank that uses cold liquid nitrogen — to preserve germplasms of plants in temperatures as low as -196 degree Celsius.

Gene banks are safe locations for germplasm — living genetic resources such as seeds or tissues of animal and plant — against catastrophe and loss in purity. These banks are also used by researchers and plant breeders. A typical gene bank would preserve seeds or vegetative tissues at extreme low humidity and temperatures.

"By keeping the moisture low, we increase the life of seeds," said Ganesh Ram, head of the department, Centre for Plant Breeding and Genetics, TNAU.

Pollen grains

Properties

- Pollen grains coated with sporopollenin – durable and chemically inert
- It is a polymer of carotenoid and carotenoid esters – difficult to disintegrate
- Pollen grains are rich in sugars, starch and other stored materials
- Important reason for preserving pollen is to use in breeding and for conservation of haploid germplasm

Objectives of Pollen storage

- It circumvents the problems of wide hybridization programs like seasonal limitations, geographical limitation and physiological limitation.
- It ensures constant supply of short lived (recalcitrant) pollen.
- It can be used for production of haploid plants.
- It could be employed in the study of pollen allergies.
- It ensures availability of pollen throughout the year.

Methods of Pollen storage

Refrigeration (3-5℃) for short term storage

- It was attempted as early as 1919 by Kihara and being used routinely.

Freeze-drying and Freeze-storage

- Pollen stored at sub-zero temperatures combined with freeze-drying (lyophilization) retain viability for years.
- E.g. Pollen grains of Rosaceae members were viable for 10 years by free-drying combined with freeze-storage at -20℃.

Methods of Pollen storage

Vacuum drying

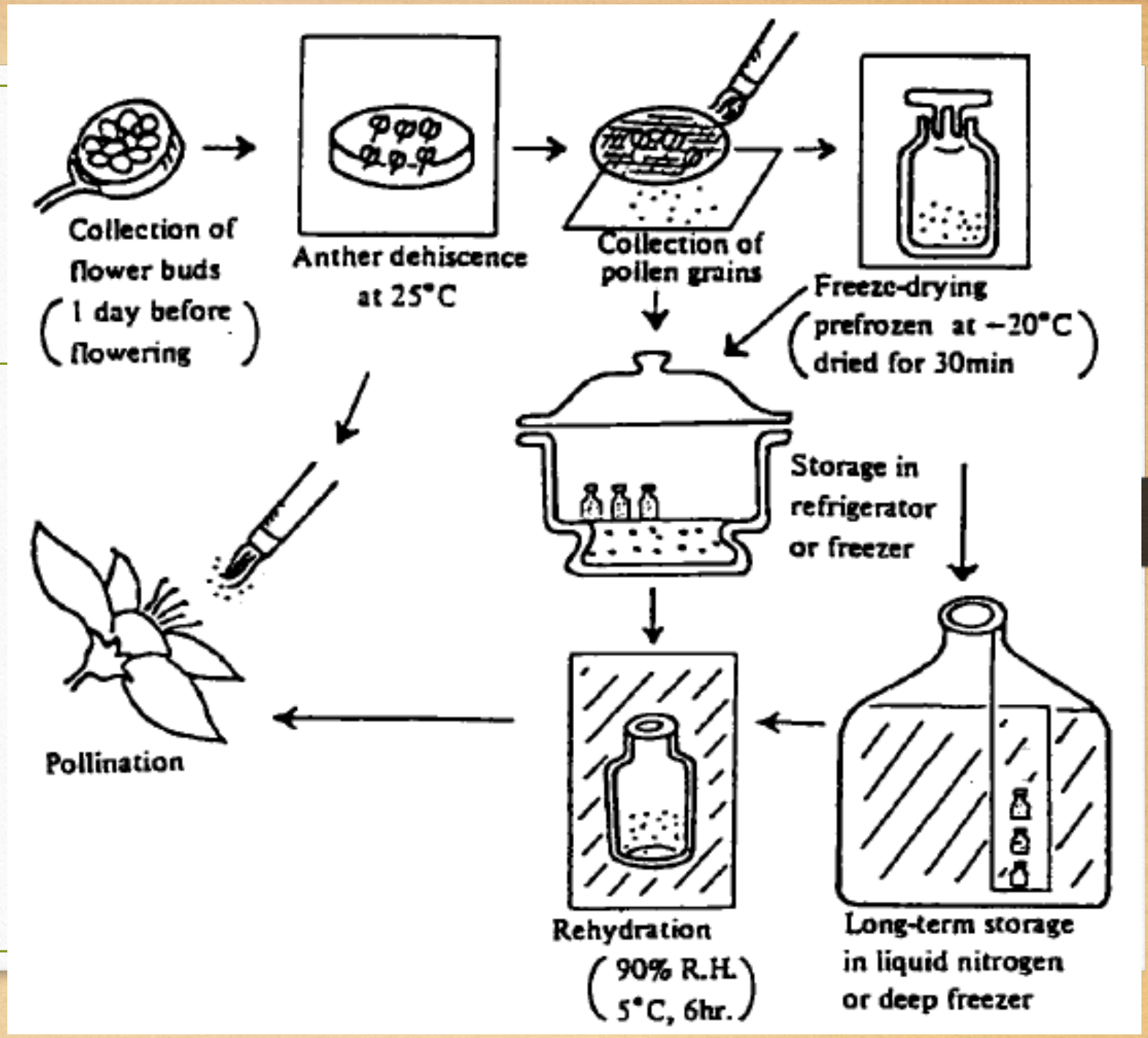
- Vacuum dried pollen grain of *Medicago sativa* was viable for 11 years upon storage at -21 ℃.

Cryopreservation of Pollen grains

Advantages

- Low water content
- Highly packed storage materials like sugars, oil and starch
- Non-vacuolated nature
- Highly resistant wall, the exine
- Do not require any specific rate of cooling.
- Can be directly immersed in liquid nitrogen.

Protocol


Collection of
flower buds
(1 day before
flowering)
Anther dehiscence
at 25°C
Collection of
pollen grains
Freeze-drying
(prefrozen at −20°C
dried for 30min)
Storage in
refrigerator
or freezer
Pollination
Rehydration
(90% R.H.
5°C, 6hr.)
Long-term storage
in liquid nitrogen
or deep freezer

Protocol

- Mature pollen grains should be collected from dehiscing anthers so that they are not ripe, not very rigid and dry.
- A sample of pollen is used to determine pollen viability by staining or in vitro germination
- Pollen does not require any treatment with cryoprotectant and hence can be maintained dry.

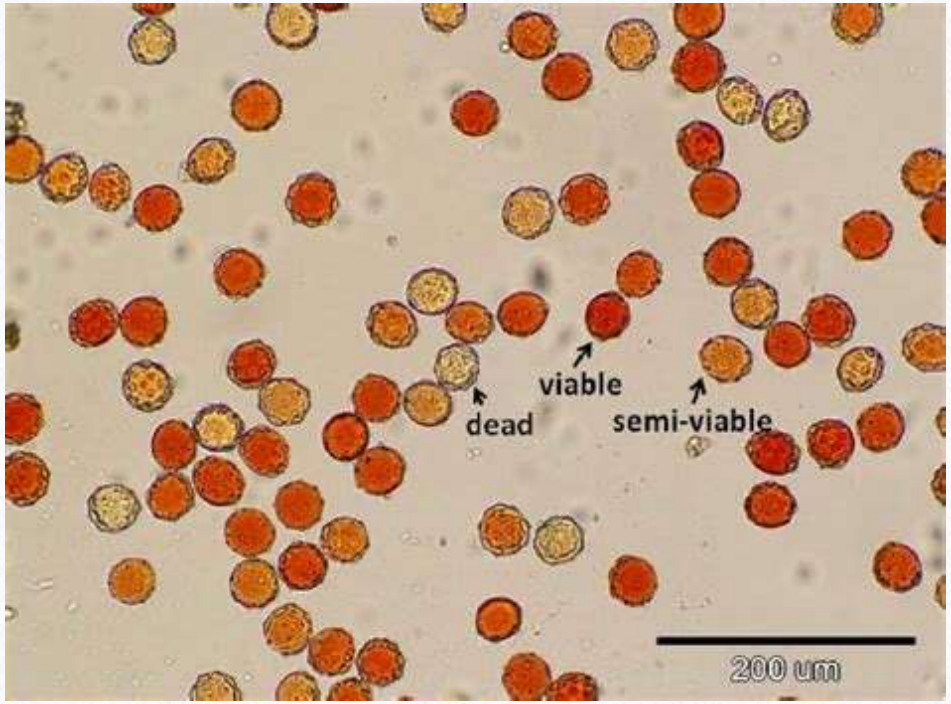
Determination of pollen viability

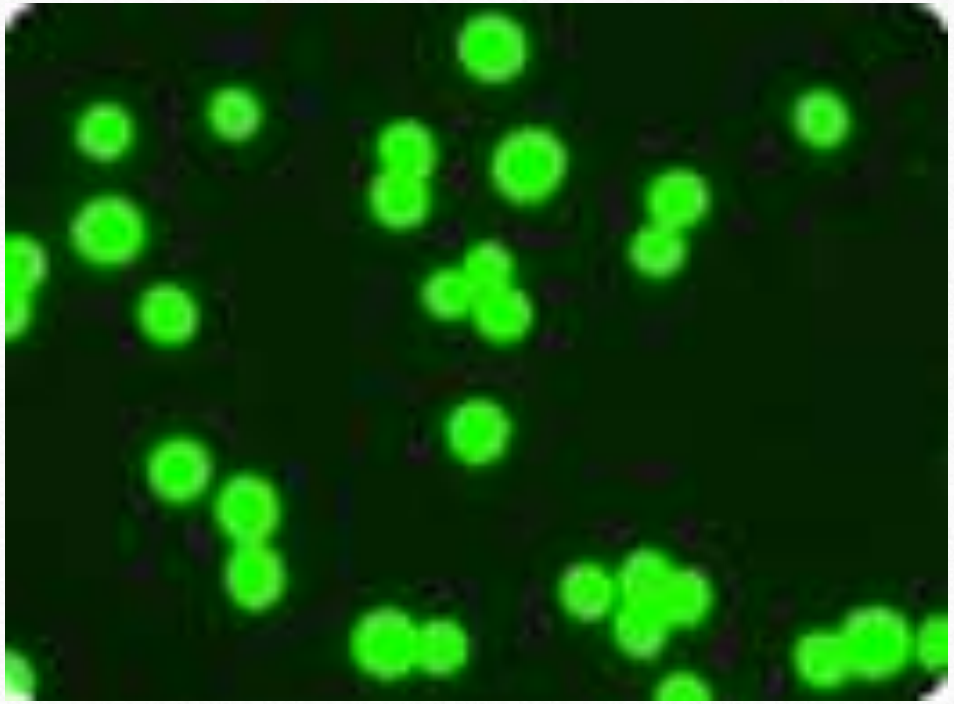
- Pollen viability was estimated using two colorimetric tests, that is, 2,3,5 triphenyl tetrazolium chloride (TTC) and acetocarmine.
- In TTC test, 0.2 g triphenyltetrazolium chloride and 12 g sucrose were dissolved in 20 ml distilled water ((Norton, 1966).
- Petri dishes containing the TTC solution were evenly dusted with fresh pollen and kept at room temperature for 2 h under daylight

Determination of pollen viability

- Both methods consist to the addition of colorant on pollen and observation under photonic microscope.
- The pollen viability was scored according to staining level (pollen with bold red colour as viable and colourless as nonviable).
- The percentage of pollen viability was determined as the ratio of the number of viable grains to the total grains number

Determination of pollen viability





Determination of pollen viability

- In FDA test, 2 mg fluorescent diacetate and 1.71 g sucrose were dissolved in 10 ml distilled water and the pollen were dusted.
- All pollen grains, which fluoresced brightly in a fluorescence microscope were scored as viable

Determination of pollen viability

- In vitro germination was assessed with the hanging drop method.
- Pollen germination and pollen tube growth were determined by placing a small drop of germinating media on a cover glass; pollen grains were sown on the drops with a clean brush, and the cover glass was then inverted and rested on the cavity slide.

Determination of pollen viability

- Pollen was incubated under dark conditions at 25 °C in a culture medium containing 5% sucrose, 5 ppm boric acid (H_3BO_3), and 1% agar for 24, 48, and 72 hours of time.
- For each incubation period, germination was recorded in three drops by counting three fields.
- A pollen grain was considered germinated when pollen tube length was at least equal to or greater than the grain diameter

Factors affecting pollen viability and storage

Moisture content and Humidity

- Lowering the moisture content and relative humidity increases viability
- Pollen grains do not survive below the threshold moisture content and relative humidity which varies according to species
- E.g. In corn pollen, relative humidity below 50% is detrimental

Factors affecting pollen viability and storage

Storage temperature

- Pollen storage at -20 °C showed reduced effectivity with the passage of time.
- Generally pollen stored at -20 °C gave normal seed set after 1 year which decreased after this time period
- However storage at -196 °C was found to superior to prolong the vibaility

Factors affecting pollen viability and storage

Nuclear condition

- Pollen grains of most of the angiosperms are binucleate. They have thick wall and resist dehydration.
- Pollen of Gramineae, Cruciferae, Araceae, Umbelliferae, Chenopoodiaceae, Caryophyllaceae are trinucleate. Trinucleate pollen are thin walled, sensitive to desiccation and storage.
- Trinucleate pollens store better at high relative humidity than binucleate pollen

Factors affecting pollen viability and storage

Method of freezing

- Rate of freezing does not affect pollen viability as they can be directly immersed in liquid nitrgogen.
- Prefreezing pollen at -15 to -30 °C for 5 hours was helpful in a number of species

Factors affecting pollen viability and storage

Oxygen/Air pressure

- Viability of pollen stored under low oxygen/oxygen pressure tend to increase

Method of thawing and Rehydration

- Rehydrating dried pollen increases germination but slow dehydration is desirable
- Rehydration at low temperature is more effective
- E.g. Hydrated tea rose pollen showed greater germination (51%) than unhydrated pollen (27%).

Factors affecting pollen viability and storage

Genotype

- Pollen of Rosaceae, Primulaceae, Saxiferaceae, Vitaceae and Pinaceae showed greater longevity (orthodox pollen)
- E.g. Clones of black walnut pollen stored in liquid nitrogen showed different germination percentage.
- Pollen of Gramineae are sensitive (recalcitrant) and viability remains from few hors to days

Factors affecting pollen viability and storage

Method of collection and physiological state of the plant

- Physiological and phytopathological status of the plant influence vigour and germinability of pollen
- Ripe and dried pollen from dehiscing anthers should be collected from vigorously growing plants with nondiseased and healthy flowers