Conservation of Plant Genetic Resources: Cryopreservation and Long-Term Storage of Genetic Material

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- The endangered and endemic species of plant biodiversity in both nature and agricultural fields have led the flora to look for alternatives to *in situ* conservation.

- Therefore, cryopreservation is a useful tool for long-term storage of plant germplasm for next generations, requiring only a minimum of space and maintenance.

- With increasing interest in the genetic engineering of plants, the preservation of cell lines (callus or protoplast) and somatic embryos with incomparable attributes is assuming highly significance.

- Latterly, cryopreservation was reported to offer real hope for enhancing the preservation of endangered and endemic plants.

Seed Banks…

- Seed banks are the world's "insurance policy" for biodiversity. While we've discussed cryobanks for delicate tissues, traditional seed banks store seeds from "orthodox" plants (those that survive drying and freezing) at around -20°C.

- There are roughly 1,700 gene banks globally, but they follow a specific hierarchy from local to global levels.

1. The Global Backup: Svalbard Global Seed Vault

Known as the **"Doomsday Vault,"** it is the ultimate safety net located on a remote Norwegian island near the North Pole.

- **Purpose:** It does not cooperate with researchers directly; it only stores **duplicates** of seeds already held in other banks.

- **Capacity:** It can hold **4.5 million varieties** (over 2 billion seeds). It currently houses over **1.3 million samples**.

- **Safety:** It is built 120 meters into a mountain, designed to withstand nuclear blasts, earthquakes, and rising sea levels. Even if power fails, the Arctic permafrost keeps the seeds frozen.

2. The Wild Plant Guardian: Millennium Seed Bank (UK)

Located at Wakehurst (part of Kew Gardens), this is the largest ex-situ collection of wild plant seeds in the world.

Focus: Unlike Svalbard, which focuses on food crops, the MSB focuses on wild species that are at risk of extinction due to climate change.

Stat: It holds over 2.4 billion seeds from nearly 40,000 species, representing about 16% of the world's wild plant species.

3. Key International Gene Banks (CGIAR Centers)

These centers focus on specific crops essential for global calories:

- ✓ IRRI (Philippines): Specializes in Rice.
- ✓ CIMMYT (Mexico): Specializes in Maize and Wheat.
- ✓ ICARDA (Lebanon/Morocco): Focuses on crops for dry regions (barley, lentils).

Note: This was the first bank to ever "withdraw" seeds from Svalbard after its original facility in Syria was damaged by war.

Feature	Seed Banks (Standard)	Cryobanks (Kriyobank)
Storage Temperature	**-20°C**	**-196°C** (Liquid Nitrogen)
Storage Material	Dried Seeds	Meristems, Embryos, Pollen
Best For	Wheat, Rice, Corn, Beans	Potato, Banana, Coffee, Oak
Duration	Decades to Centuries	Theoretically Indefinite

Why are Seed Banks failing?

Despite their importance, many national seed banks are at risk due to:

Lack of Funding: Maintenance of cooling systems is expensive.

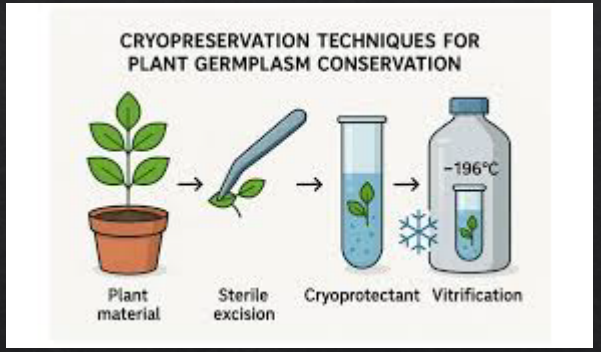
Natural Disasters: Floods or fires can destroy unique genetic heritage.

War: As seen in Syria and Iraq, conflict can wipe out decades of agricultural research.

What is Cryopreservation?

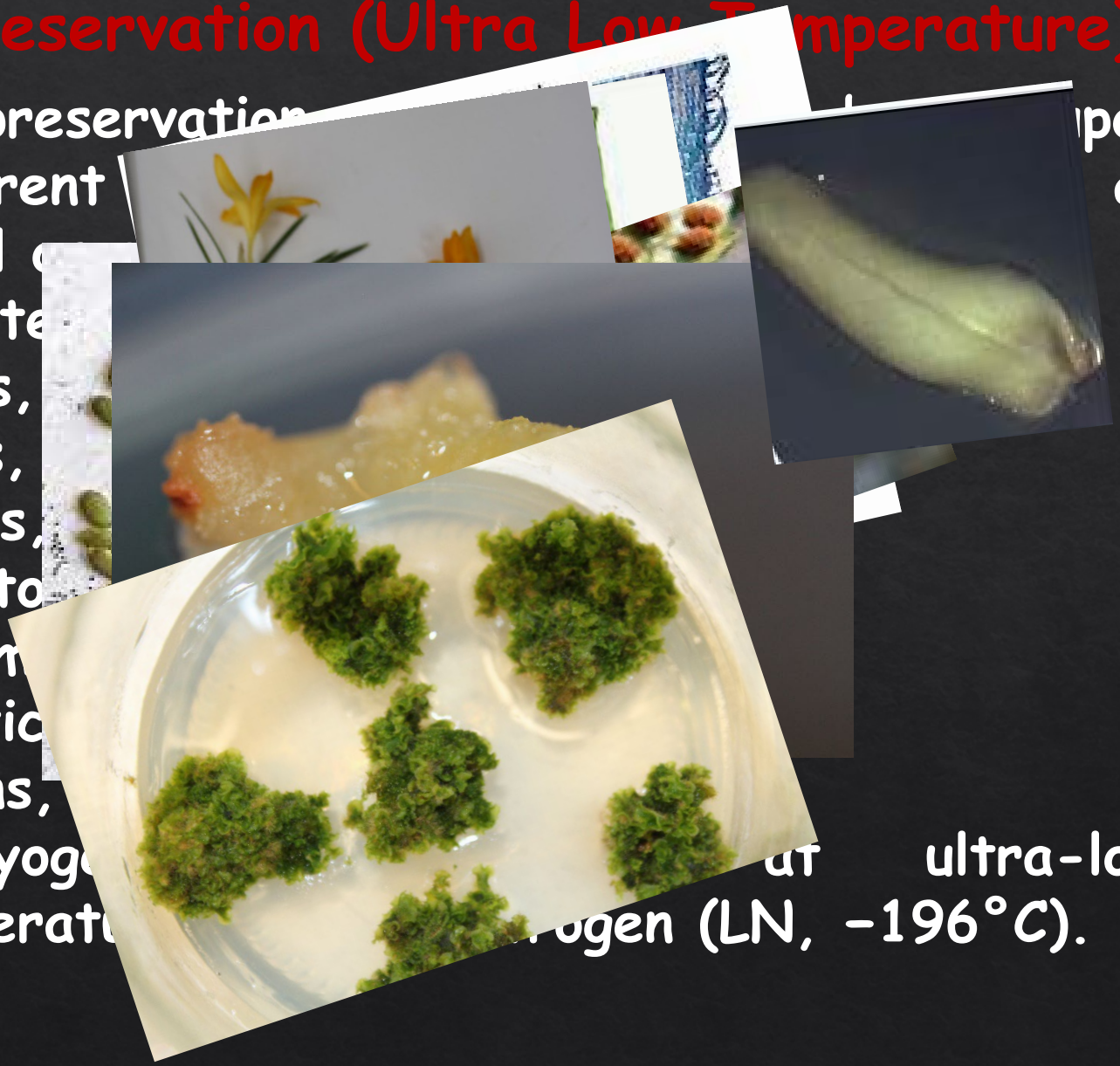
Cryopreservation is a scientific process that involves preserving biological materials—such as cells, tissues, or organs—by cooling them to ultra-low temperatures, typically **-196°C** using **liquid nitrogen**.

At these temperatures, all metabolic and biological activities inside the cells come to a complete halt. This essentially "freezes time," allowing biological samples to be stored for decades or even centuries without degrading.

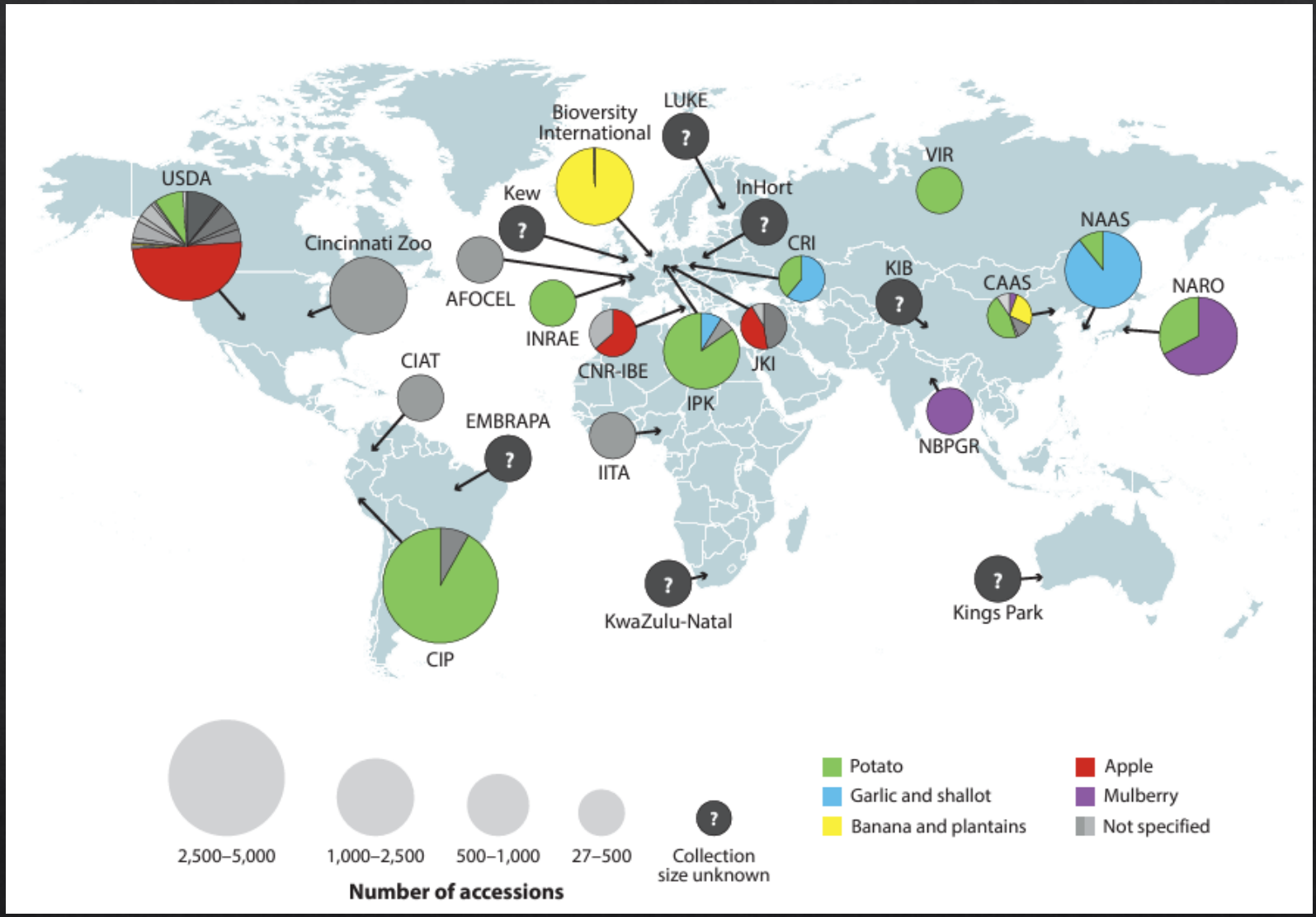


Cryopreservation (Ultra Low Temperature)

- ✓ Cryopreservation … upon different … as apical …
  - ❑ meriste…
  - ❑ scions,
  - ❑ seeds,
  - ❑ spores,
  - ❑ gameto…
  - ❑ rhizom…
  - ❑ zygotic…
  - ❑ pollens,
  - ❑ embryog… at ultra-low temperat… ogen (LN, −196°C).



- ✓ The major advantage of this procedure is to diminish in vitro culture costs, required space, contamination and somaclonal variation or genetic altering risk.
- ✓ The long-term conservation of embryogenic cell lines may be a valuable tool in genetic engineering.



Nagel, M., Pence, V., Ballesteros, D., Lambardi, M., Popova, E., & Panis, B. (2024). Plant cryopreservation: Principles, applications, and challenges of banking plant diversity at ultralow temperatures. *Annual Review of Plant Biology*, *75*.

Institution / Center	Location	Primary Focus / Species
CIP (International Potato Center)	Lima, **Peru**	World's largest collection of **potato, sweet potato**, and Andean root crops.
ITC (Musa Germplasm Transit Centre)	Leuven, **Belgium**	Global **banana and plantain** collection (managed by Bioversity International).
CIAT (Future Seeds)	Cali, **Colombia**	**Beans, cassava**, and tropical forages.
IPK Gatersleben	**Germany**	**Garlic, onion**, and various European temperate fruit trees.
IITA	Ibadan, **Nigeria**	**Yam, cassava**, and African banana/plantain varieties.
USDA-NCGRP	Fort Collins, **USA**	Fruit trees, medicinal plants, and rare/endangered wild species.

Institute	N° of Acc.	Crop	Cryopreservation Method
Bioversity International, Leuven, Belgium	1100	Banana	• Droplet vitrification
Association FOrêt-CELlulose (AFOCEL), France	440	Elm	• Dormant bud freezing
International Center for Tropical Agriculture (CIAT), Cali, Colombia	480	cassava	• Droplet vitrification • Encapsulation/dehydration
Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben, Germany	213	Garlic	• Droplet vitrification
International Potato Center (CIP), Lima, Peru	3227	Potato	• Droplet vitrification
Julius Kühn-Institut (JKI), Institut für Züchtungsforschung an Obst, Dresden, Germany	194	Strawberry	• Vitrification
Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben, Germany	1818	Potato	• Droplet freezing • Droplet vitrification
National Agrobiodiversity Center (NAAS), RDA, Suwon, South Korea	1158	Garlic	• Droplet vitrification
National Institute of Agrobiological Sciences (NIAS), Tsukuba, Japan	1236	Mulberry	• Dormant bud freezing
USDA-ARS, Fort Collins and Corvallis, USA	2155	Apple	• Dormant bud freezing
USDA-ARS, Fort Collins and Corvallis, USA	451	Citrus	• Droplet vitrification
Tissue Culture and Cryopreservation Unit, NBPGR, Delhi, India	329	Mulberry	• Dormant bud freezing
Crop Research Institute, Prague, Czech Republic	157	Garlic	• Droplet vitrification

https://www.ecpgr.org/working-groups/cryopreservation

The **CNR-IBE (Institute of BioEconomy)** in Sesto Fiorentino, Florence, hosts one of Italy's most significant and pioneering plant cryobanks. It is a center of excellence for the long-term conservation of fruit tree genetic resources.

1. The Collection (What is Preserved?)

The cryobank focuses on the "ex-situ" conservation of ancient and commercially important fruit tree germplasm that cannot be easily stored as standard seeds.

•**Citrus Collection:** Perhaps the most famous part of the bank. It preserves polyembryonic seeds from **24 accessions of *Citrus spp.*** dating back to the **16th-century Medicean Collection** of Villa Reale di Castello in Florence.

•**Ancient Fruit Varieties:** * **Apple (*Malus domestica*):** Dormant buds from over 25 ancient varieties, particularly from the Veneto region and Tuscany.

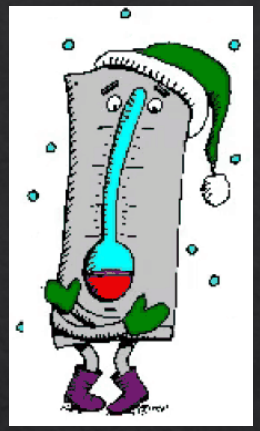
- **Plum (*Prunus domestica*):** Several cultivars of European and Sino-Japanese plums.
- **Olive & Pear:** Ongoing research and collections for Mediterranean woody species.

•**Wild & Endangered Species:** Specialized projects like the conservation of the **Sicilian Fir (*Abies nebrodensis*)**.

Techniques of Cryopreservation

- Cryopreservation techniques are separated in to two main groups: traditional (two step freezing) and modern (one step freezing) techniques. There are several combinations of the cryopreservation techniques.

- The combinations of these techniques are now directly applicable for many plant species. These techniques:



I. Controlled rate cooling (two-step freezing, controlled-rate freezing or slow cooling)

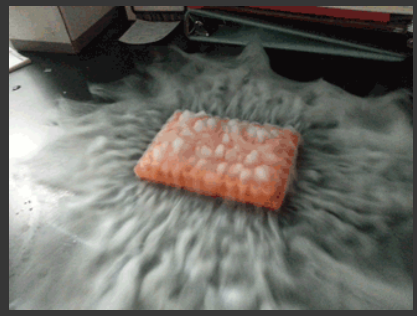
II. Vitrification (with plant vitrification solution-2 "PVS2") (Sakai et al. 1990)

*PVS2 including 30% glycerol (w/v) + 15% ethylene glycol (w/v) + 15% dimethylsulfoxide (DMSO; w/v) in MS (Murashige and Skoog, 1962) basal medium (plant growth regulators free) containing 0.4 M sucrose (pH 5.8)

III. Encapsulation/vitrification

IV. Encapsulation/dehydration

V. Droplet method



VI. Dessication (Laminal flow cabinet or silica gel)

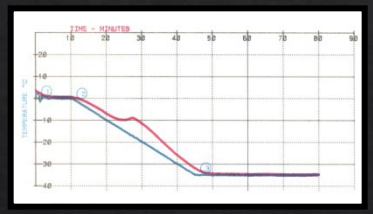
VII. V-Cryo-plate and D-Cryo-plate procedures

Traditional methods: Two step freezing (Slow Freezing)

Modern methods: One step freezing (Quick freezing)

Slow freezing

(per minute -0.5/-1°C)



PVS2 with vitrification

30% glycerol, 15% etylenglycol, 15% DMSO, MS + 0.4M sucrose (Sakai et al., 1990)

Dehydration – encapsulation/ dehydration

silica gel or laminal flow cabinet

Pyhsical dehydration

Cryopreservation of Cells

LN Storage

- ✓ Slow cooling is provided by a device named "programmable freezer". In this system, the parameters can be adjusted easily such as starting temperature, freezing rate, final temperature and waiting time.

- ✓ Because this method is expensive, lower technology (Nalgene freezing container, Mr. Frosty®) can be used in stead of it.

- ✓ Isopropanol contained in a Mr Frosty unit provides a 1° $C \cdot min^{-1}$ cooling rate (–20 or –80° C). Followed by a direct plunge into LN.

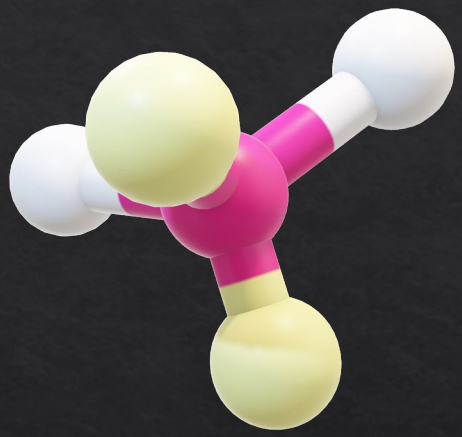

NALGENE™ Cryo 1°C Freezing Container, Cat. No. 5100-0001
To achieve a -1°C/min. rate of cooling:
NOTE: Store at room temperature when not in use. Replace alcohol after every fifth use.
1.2 mL
2.0 mL


NALGENE™ Cryo 1°C Freezing Container, Cat. No. 5100-0001
To achieve a -1°C/min. rate of cooling:
NOTE: Store at room temperature when not in use. Replace alcohol after every fifth use
IPA
(C3H8O)
250 mL
1.2 mL
2.0 mL

The Biological Chemistry of Water

Water, nature's biological solvent, is highly influential in determining survival after exposure to liquid nitrogen (LN).

Ice Nucleation!!!



It is popularly believed that water freezes at 0°C, but this is rarely the case. In the absence of templates that allow the coming together of H_2O molecules, water super-cools to freezing points below zero.

The lowest possible supercooling temperature in most biological systems is the point of homogeneous ice nucleation, around or at -40°C.

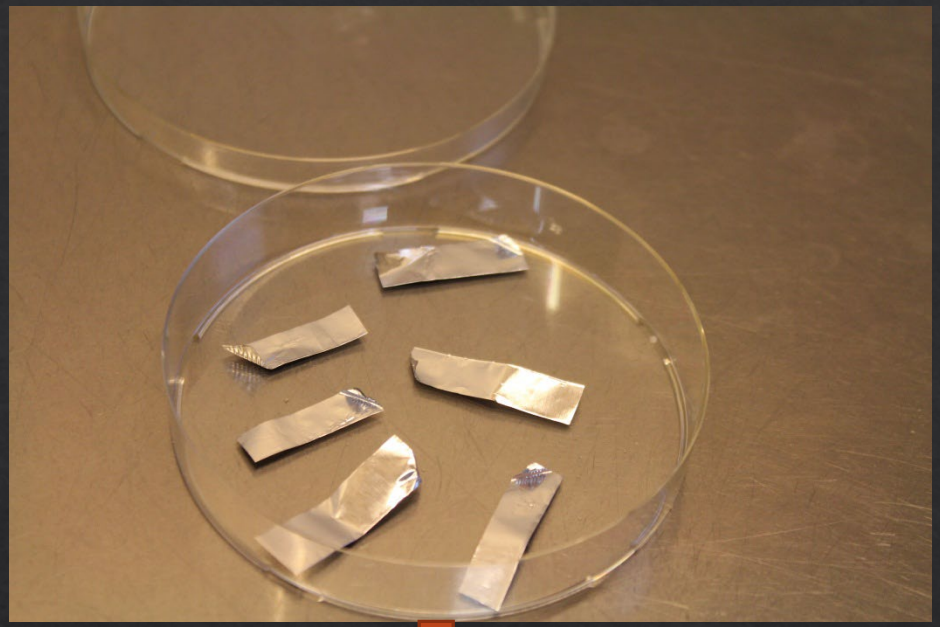
At this temperature, water molecules form an "ice embryo" of a critical size that is thermodynamically capable of growing a crystal. This creates an ordered matrix and energy is released as the latent heat of fusion and as ice is formed heat is produced.

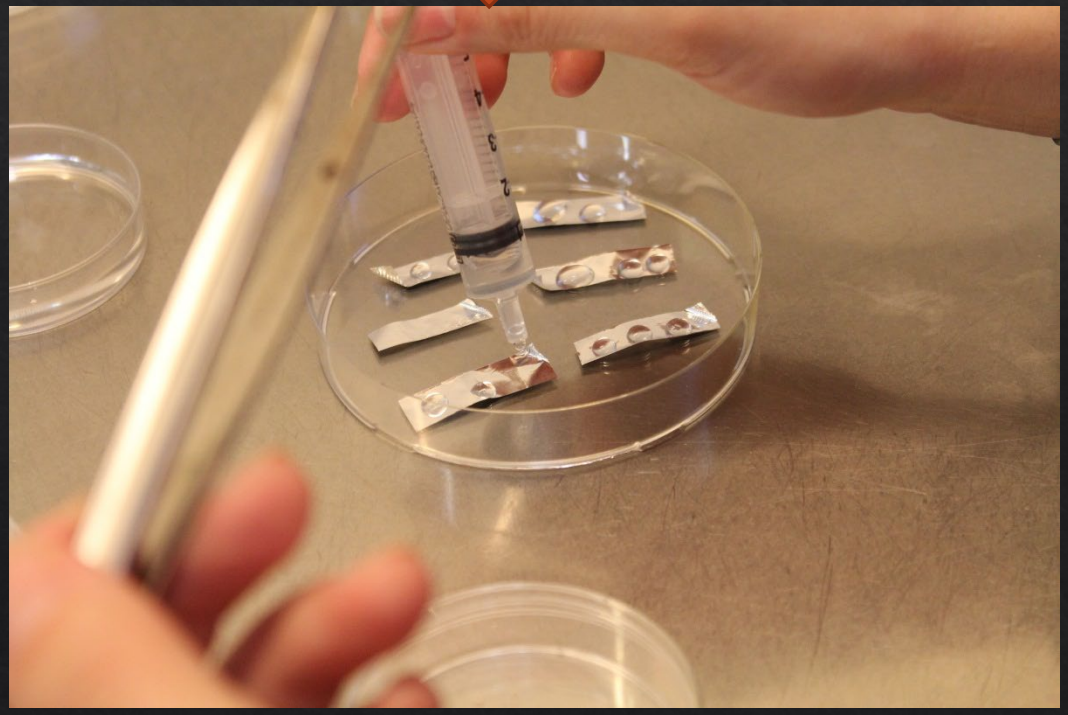

DANTE ALIGHIERI
"DIVINE COMEDY"
-INFERNO
-PURGATORY
-HEAVEN
ARE THEY DEAD OR ALIVE??
Eternal hibernation!!!

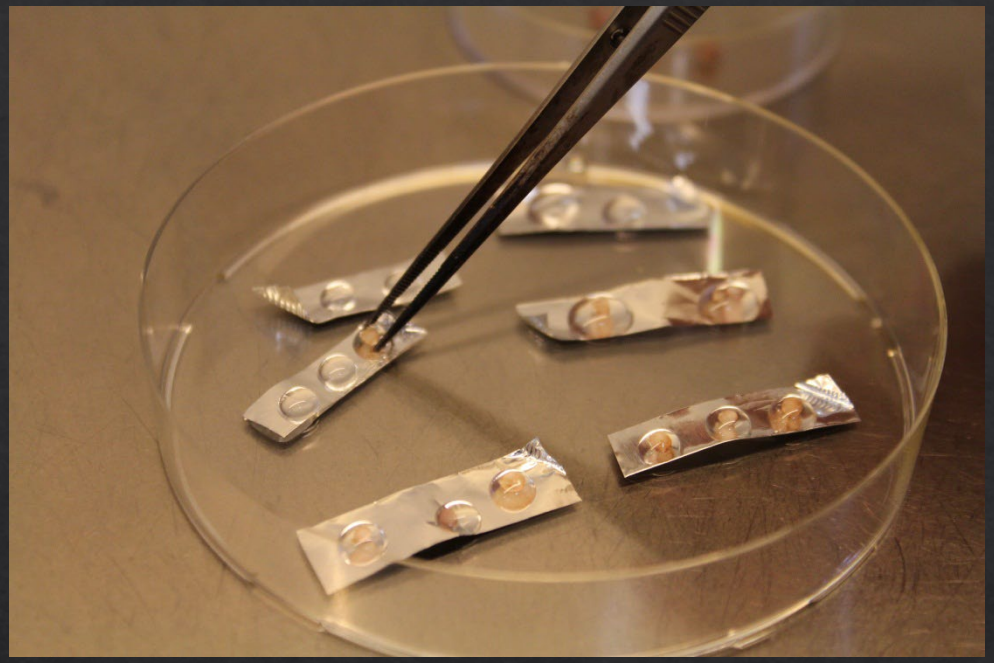
Vitrification

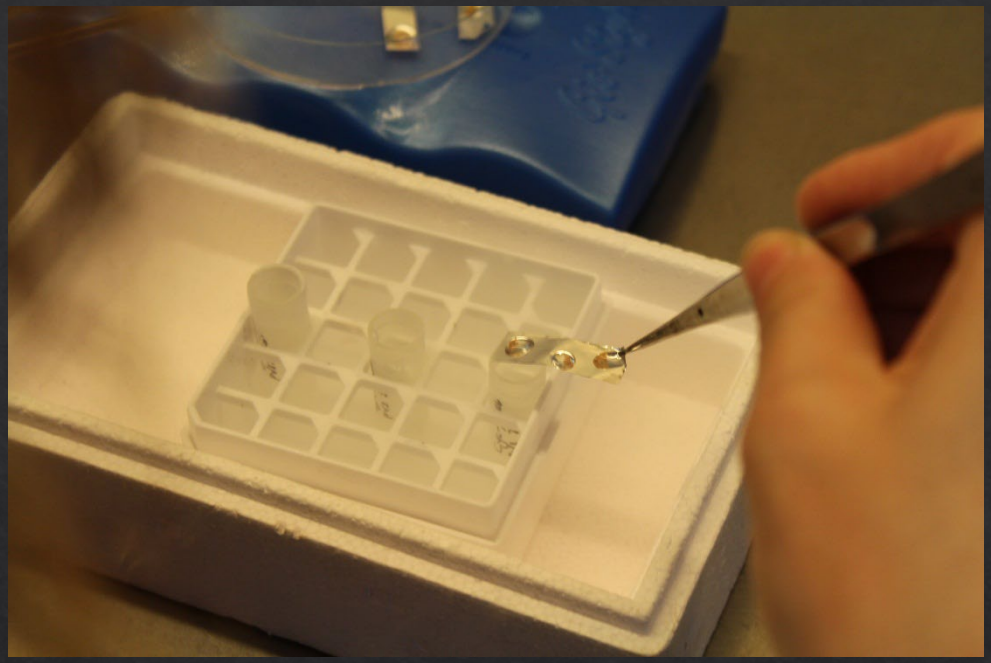
- Vitrification is very effective method for the cryopreservation of plant materials because it would avoid the potentially impairing effects of intracellular and extracellular freezing.
- This method can be defined as the transition of water directly from the liquid phase into an amorphous phase, while avoiding the formation of crystalline ice.
- Vitrification is commonly used for embryogenic callus lines, meristems, somatic embryos and etc. in LN.
- In vitrification methods, embryogenic callus/somatic embryos can be sufficiently dehydrated with PVS2 at 25°C or 0°C without causing injury to enable vitrification upon rapid cooling in LN.

Droplet Method










Dehydration Cryopreservation
Silica gel

Encapsulation-Synthetic seeds with Na-alginate


MS+0,7H / Callus / Cyclamen
11/05/12
III


2.0
1.5
ml
1.0

Aluminium V-D Cryoplate

A new cryopreservation methods based on vitrification and air dehydration of explants placed on aluminum cryo-plates, named the V-cryo-plate and D-cyo-plate technique.

Recently, a vitrification protocol using the aluminum v-cryo-plate method has been reported (Niino et al. 2014; Yamamoto et al. 2012a, b; Sekizawa et al. 2011; Yamamoto et al. 2011 a, b).

The cryo-plate method has two main advantages:

(i) a user-friendly procedure and,

(ii) higher cooling and warming rates of treated explants are possible by directly immersion in LN (Niino et al. 2014).

Consequently, efficient regrowth was obtained after cryopreservation of the various plant explants (Niino et al. 2013).

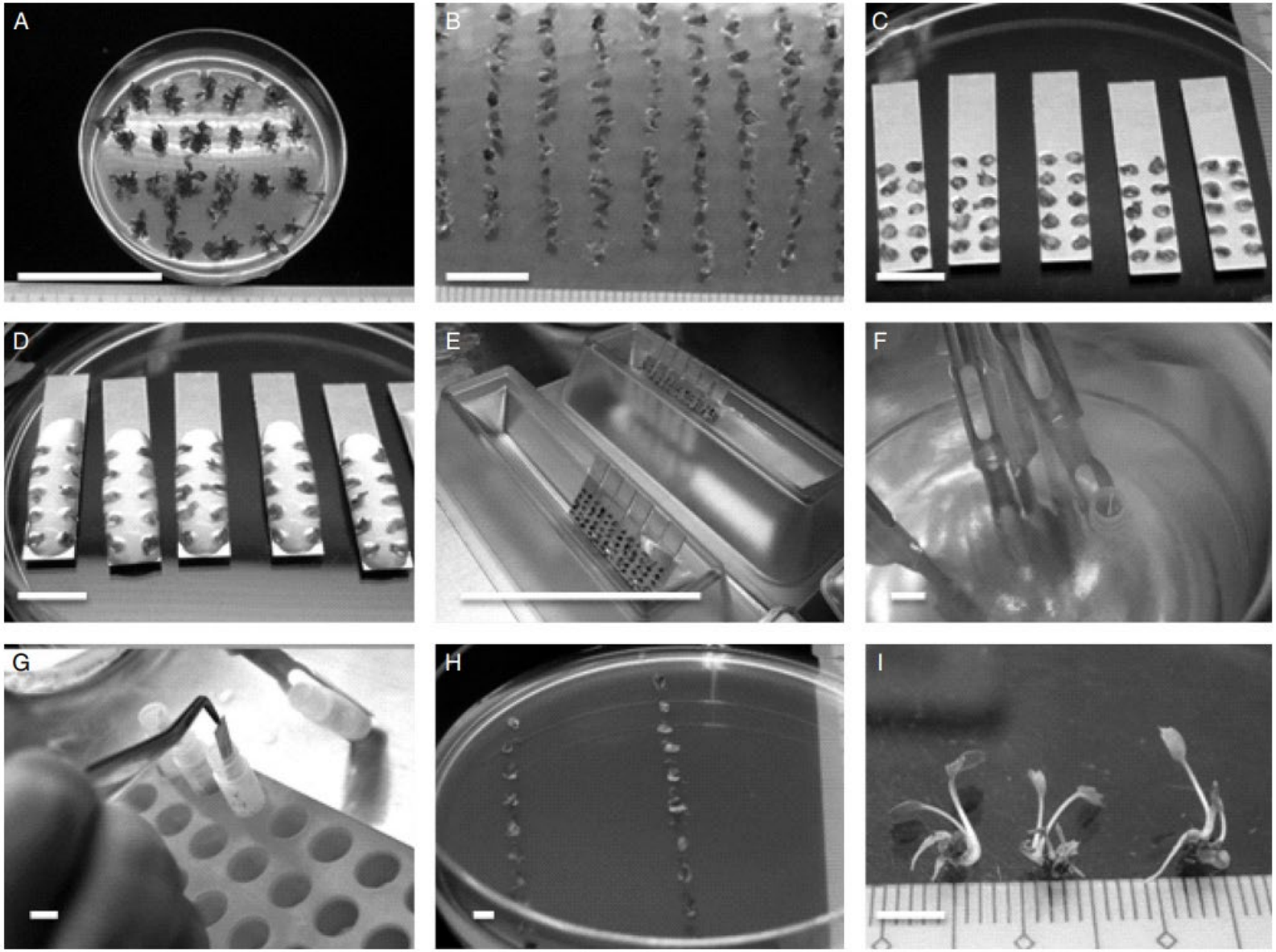
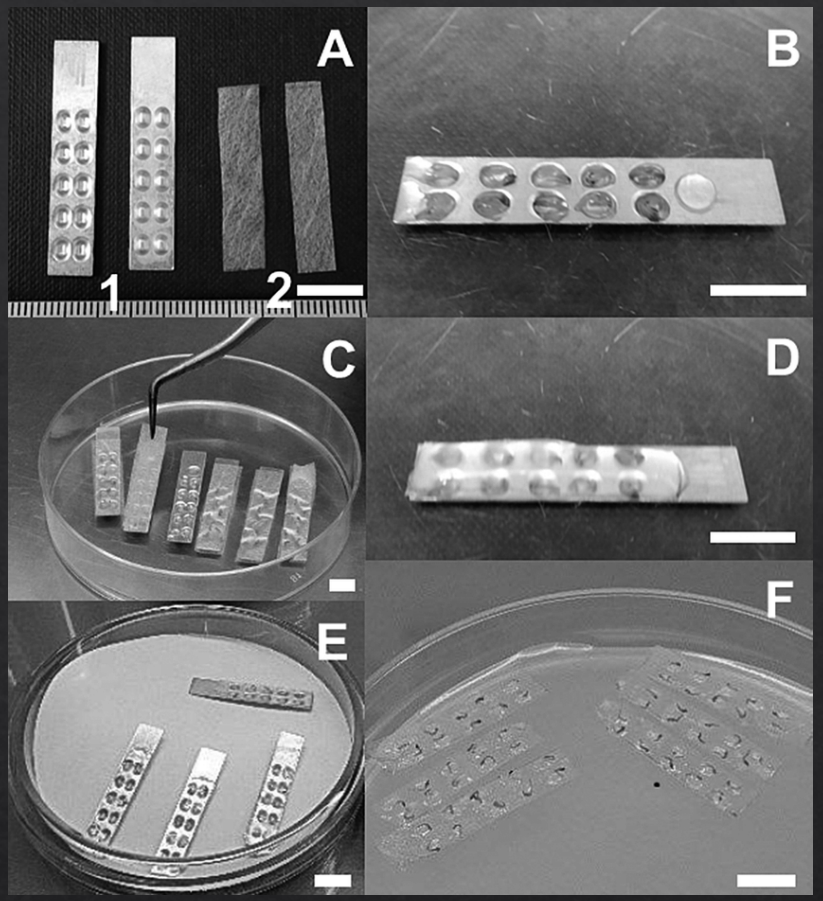
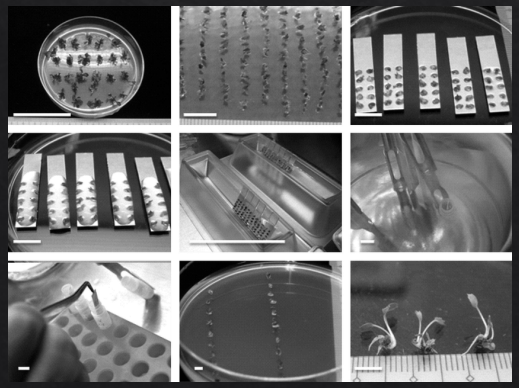


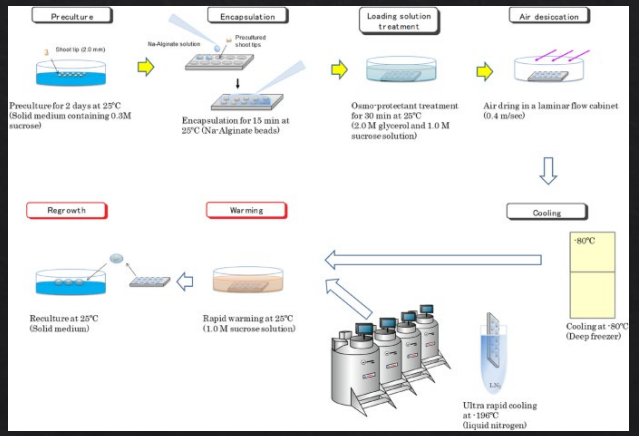
Fig. 1. The V-Cryo-plate procedure and appearance of *in vitro*-grown strawberry after cryopreservation. (A) Shoot after cold-hardening, (B) pre-culture on the MS medium with 0.3 M sucrose and 2 M glycerol after excision, (C) shoot tips mounted on the aluminium plates, (D) hardening of the alginate gel, (E) treatment by LS (left) and PVS2 (right), (F) storage of cryotubes on the canes in LN, (G) removal from LN and warming in 1 M sucrose solution (2 ml), (H) plating the vitrified shoot tips in the gel, (I) regenerated plantlets 20 d after plating (cultivar Cavalier). Scale bars indicate 50 mm (A, E), 5 mm (B–D, F, G, I) and 1 mm (H).

- In the D cryo-plate method, shoot tips or buds attached to the cryo-plates are dehydrated in the laminar air flow cabinet or over silica gel after loading treatment with glycerol and sucrose solution for inducing tolerance to dehydration.

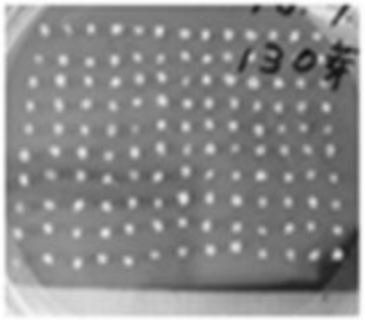
- Capacious samples consisting of buds and basal stems can be used for the materials in this technique. So it is a practical and efficient method for cryopreservation (Niino et al., 2014).







D cryo-plate method



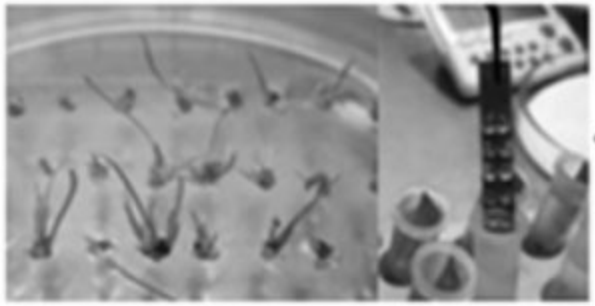
Excision shoot tips, Preculture

Adhere the shoot tips on cryo-plate

Osmoprotection by LS

Dehydration by laminar air flow in cabinet or by silica gel in a Petri dish

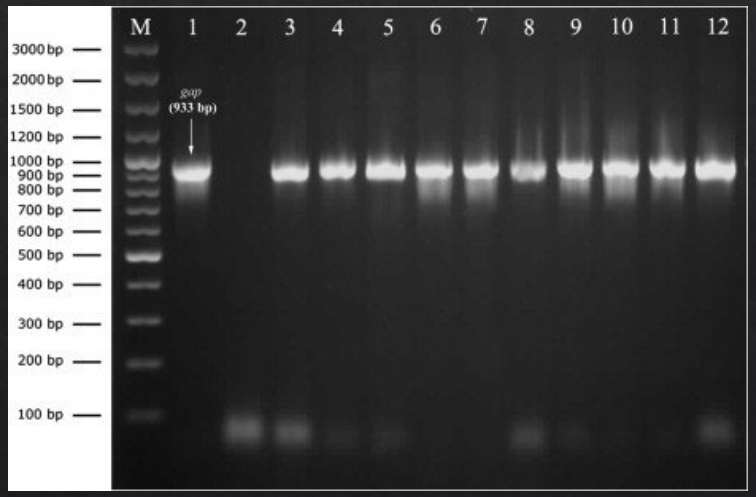
Immersion into LN



Rewarm and Plating

Procedure of D cryo-plate method (Niino et al., 2013, 2014).

Determination of Vitality, Genetic Stability



- TTC test for viability

- Germination Test

- Cryogenic procedures mutagenic? → Both Yes and No!!

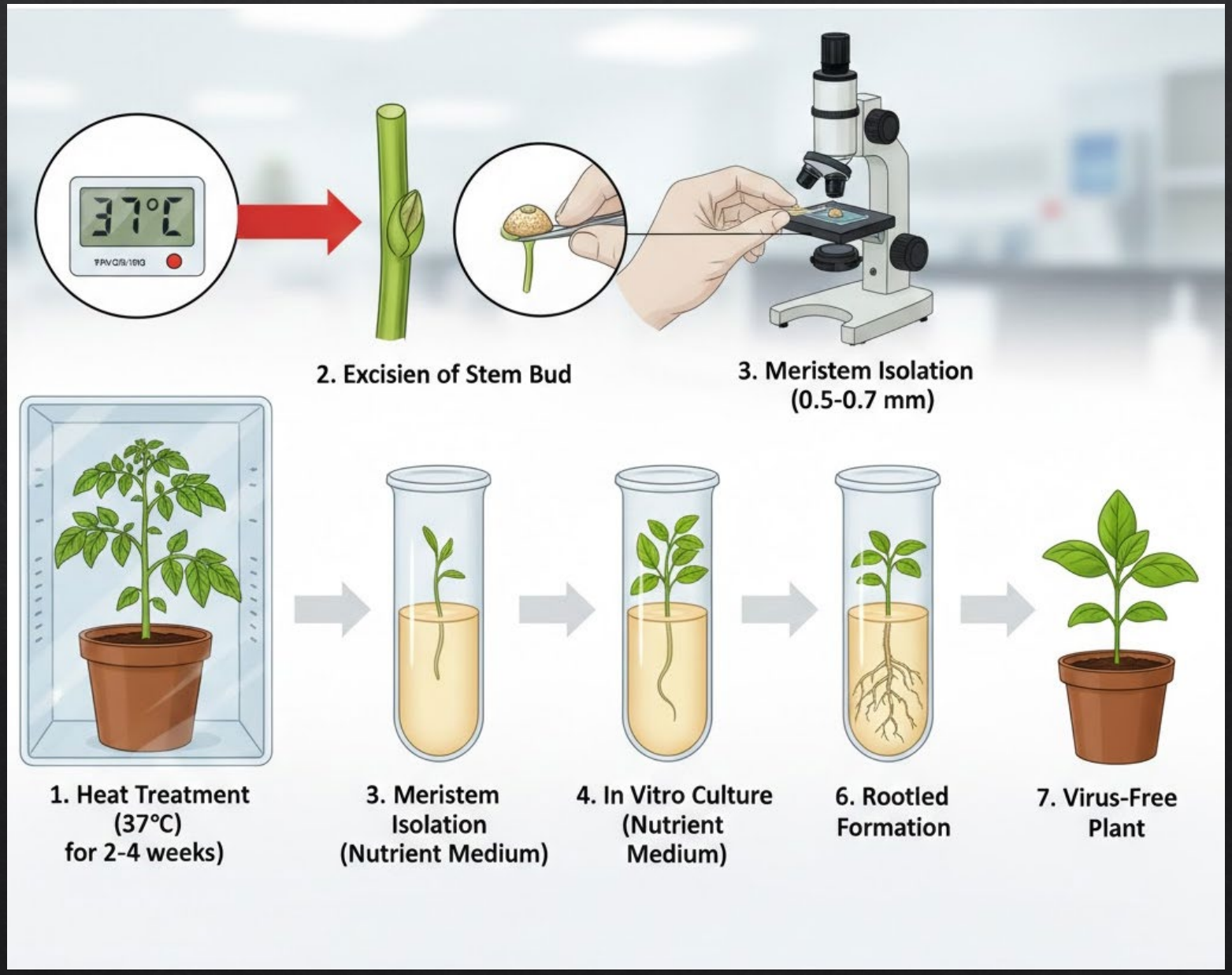
- Genetic Stability
  - Based on PCR, (RFLP, RAPD, AFLP, SSR…)
  - Based on DNA hybridization → DNA, RNA sequencing
  - Somaclonal variation !!!

Other Usage of Cryopreservation

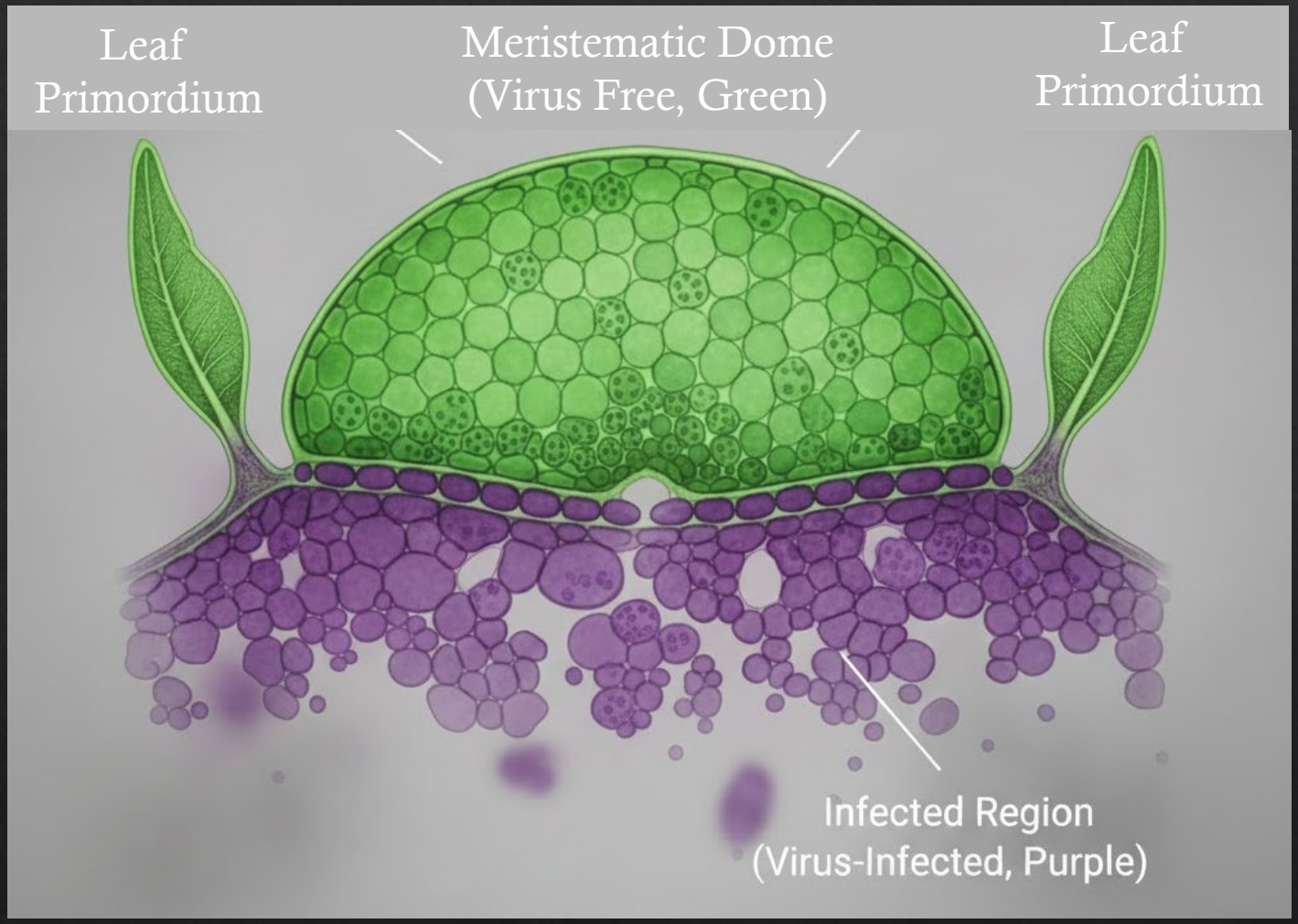
Cryotherapy !!

- ✓ In recent years, cryopreservation has also been used for cryotherapy, that is, as a complement to classical virus elimination procedures such as meristem culture or thermotherapy in virus elimination from infected cultures.

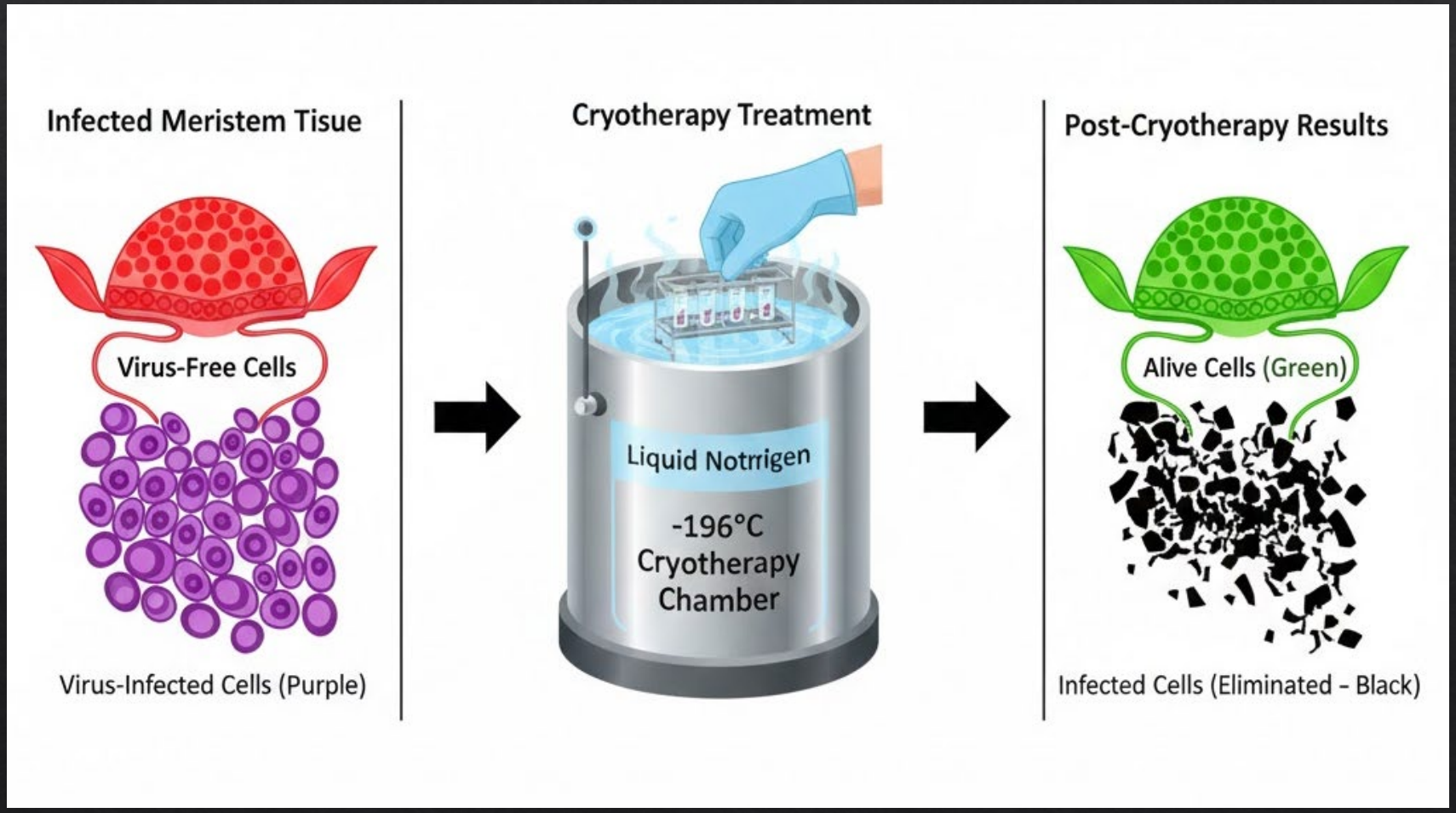
Classic approach of virus elemination



Cell Type	Characteristics	Outcome
Virus-Infected Cells	Large, high water content, large vacuoles.	**Plasmolysis/Rupture:** Water turns into ice crystals and causes the cell to burst.
Meristematic Cells	Small, low water content, dense cytoplasm/proteins.	**Survival:** Instead of crystallization, **vitrification** (glass-like state) occurs.


Leaf Primordium
Meristematic Dome (Virus Free, Green)
Leaf Primordium
Infected Region (Virus-Infected, Purple)

What's happening in Cryotherapy…



Somatic embryogenesis, Cryopreservation, Cyclamen Sp.

Cryopreservation-Vitrificaion

Expreminet of Cryopreservation

Cold Hardening

(2 weeks, +4°C, Darkness)

Preculture

a) 2% sorbitol+MS (hf) (48h, +4°C, darkness)
b) 4% sorbitol+MS (hf) (48h, +4°C, darkness)
c) 2% mannitol+MS (hf) (48h, +4°C, darkness)
d) 4% mannitol+MS (hf) (48h, +4°C, darkness)
e) 2% sobitol+2% mannitol+MS medium (hf) (48h, +4°C, darkness)
f) 0,5M sucrose+MS (hf) (48h, +4°C, darkness)

Cryopreservaton Vitrification

1- Cryoviol (embrygenic callus and microtuber)
* 30 min, 25°C, Loading solution
2- PVS-2 (Plant vitrifikasyon solüsyonu-2) adding
(PVS2; 30% gliserol (w/v) + 15% Etylen glycol (w/v) + 15% DMSO (w/v)
+ 5% sucrose (w/v))
*30 min, 0°C /+4°C
*60 min, 0°C /+4°C
*90 min, 0°C /+4°C

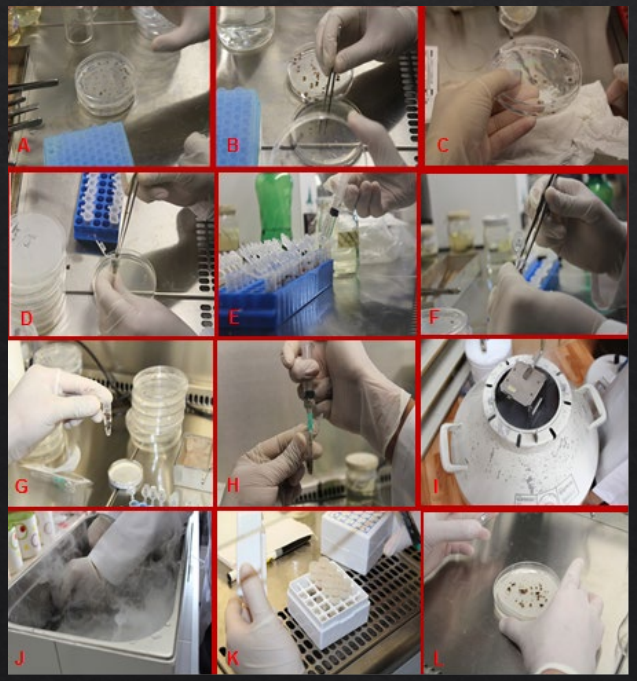
Plunged in LN (-196°C)

(at least 1 h)

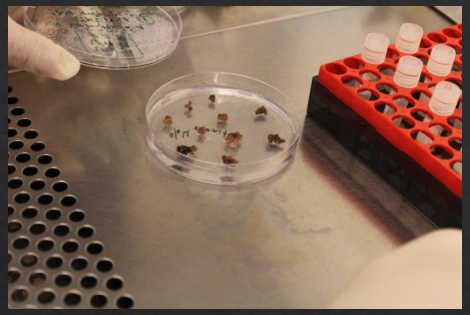
Water bath

Thawing 37°C, 3 min.

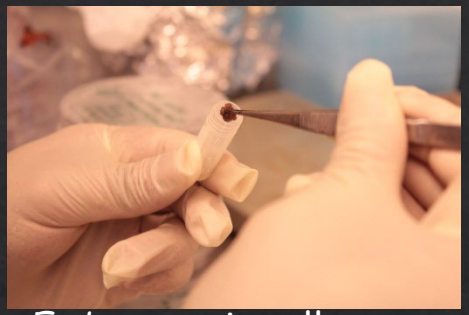
TTC test



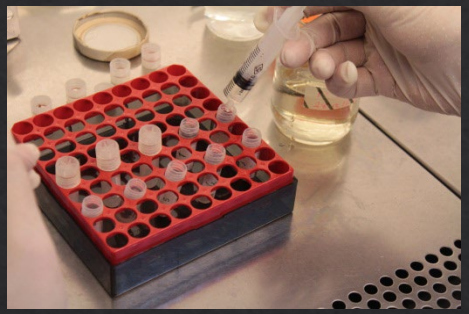
Steps of Cryopreservation



Preculture on 0.5 M sucrose



Embryogenic callus transfered to cryoviols



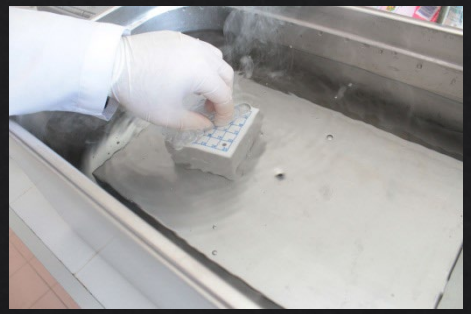
Adding of Loading solution

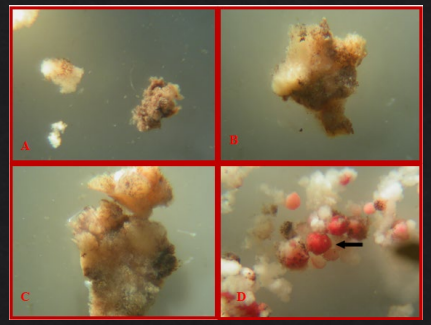
Adding PVS2 solution

0°C, 1 h

Plunged in LN (-196°C)

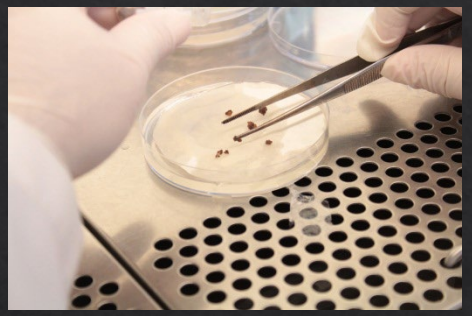
Thawing (37°C, 3 min)



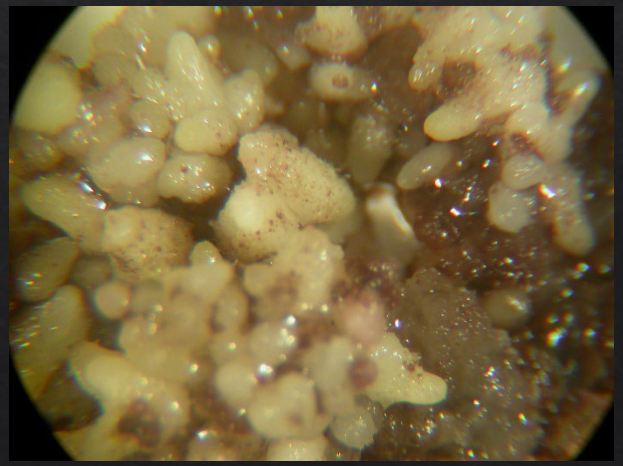


TTC test

Transferred to Regeneration Medium



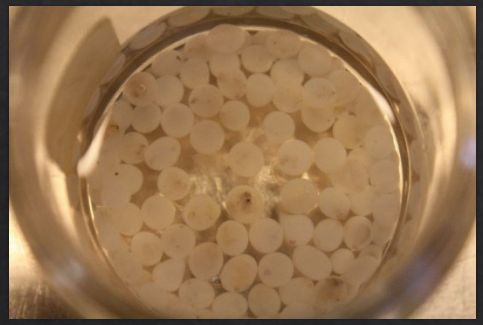
10 weeks later



Somatic embryos

Cyclamen plantlets growing after cryopreservation

Encapsulation Cryopreservation



Synthetic seeds

Preperation of samples

Adding PVS2 solution

PVS-2 at 0°C

Transferred

Plunged in LN (1h)

Thawing for 37°C at 3 min

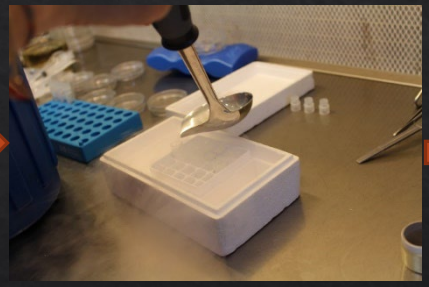
Cultivation After Thawing

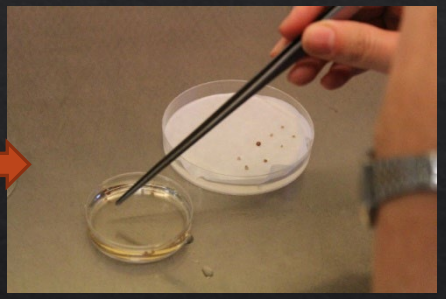
Droplet Method




Placement of explants in PVS-2 droplets on aluminum foil

25 min at 0°C

Direct thawing

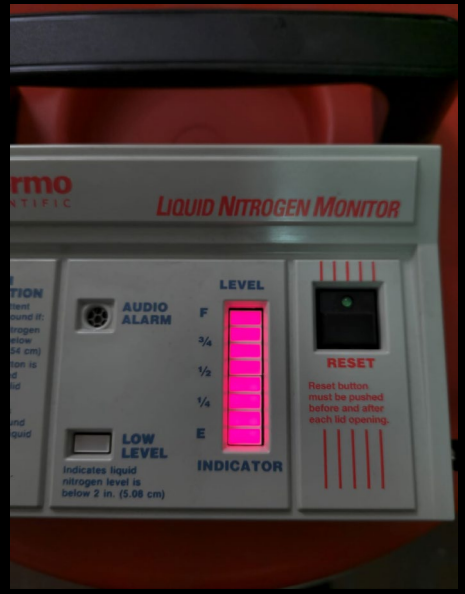
Dehydration-Cryopreservation on Silica gel and Laminar Flow Cabinet

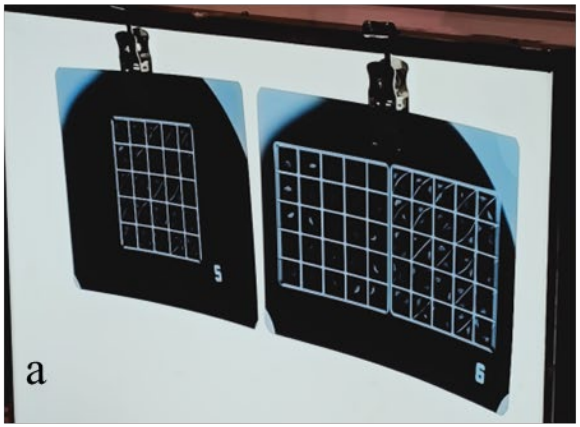
- *Abies nebrodensis*, commonly known as the Sicilian fir, is a critically endangered conifer species endemic to the Madonie Regional Park in Sicily, Italy.
- The relic population is limited to 30 relic adult trees, this species faces significant threats from genetic erosion, habitat fragmentation, and poor natural regeneration. *Abies nebrodensis*, like other conifers, produces many seeds without embryos.
- For effective conservation, it is essential to select only full seeds containing complete embryos. Conservation efforts for *A. nebrodensis* are crucial to prevent its extinction and preserve its genetic diversity.
- For ex situ conservation of this species, both through a Seed bank (medium-term preservation) and Cryobank (long-term preservation), that are established in the Museum of *Abies nebrodensis* (MAN) in Polizzi Generosa (Palermo), Italy.
- This strategy developed for *A. nebrodensis* can be a way for similar initiatives for other critically endangered conifer species.

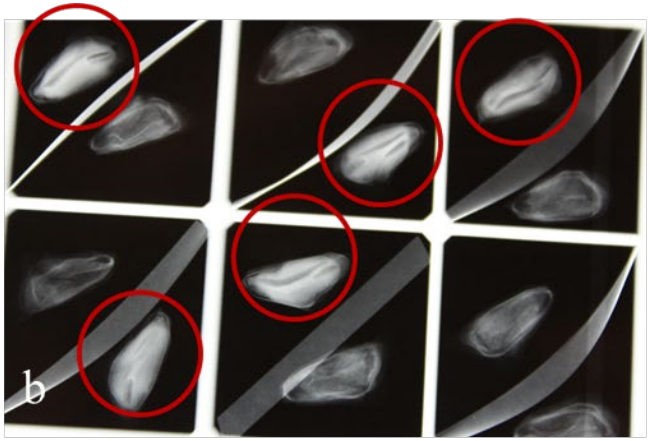

LIFE 4FIR
NATURA 2000
Progetto LIFE4FIR
LIFE18 NAT/IT/000164
«Strategie innovative di conservazione dell'Abies nebrodensis»
«Decisive in situ ed ex situ conservation strategies to secure the critically endangered Sicilian fir. Abies nebrodensis»
Banca del Seme e Criobanca
Seed Bank and Cryobank
Istituto per la Protezione Sostenibile delle Piante
Consiglio Nazionale delle Ricerche
IPSP
Consiglio Nazionale delle Ricerche
Istituto per la BioEconomia
Dipartimento di Scienze Bio Agroalimentari
Parco delle Madonie
SAAF
UNIVERSITÀ DEGLI STUDI DI PALERMO
www.life4fir.com
LIFE4FIR


ATTENZIONE
ASFISSIANTE
AZOTO
LIQUIDO


Thermo
LIQUID NITROGEN MONITOR

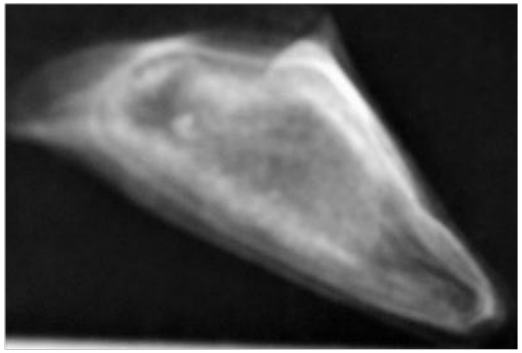

LIQUID NITROGEN MONITOR
AUDIO ALARM
LOW LEVEL
Indicates liquid nitrogen level is below 2 in. (5.08 cm)
LEVEL
F
¾
½
¼
E
INDICATOR
RESET
Reset button must be pushed before and after each lid opening.



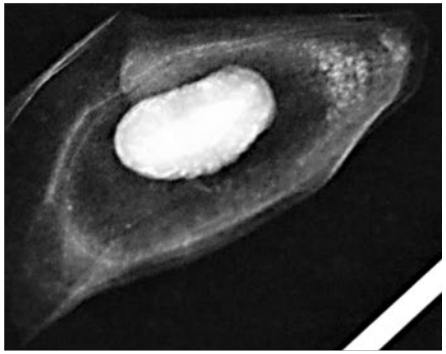


a. Checking of the seeds on X-Ray film viewer Screen
b. Empty seeds and full seeds (in the red circle)

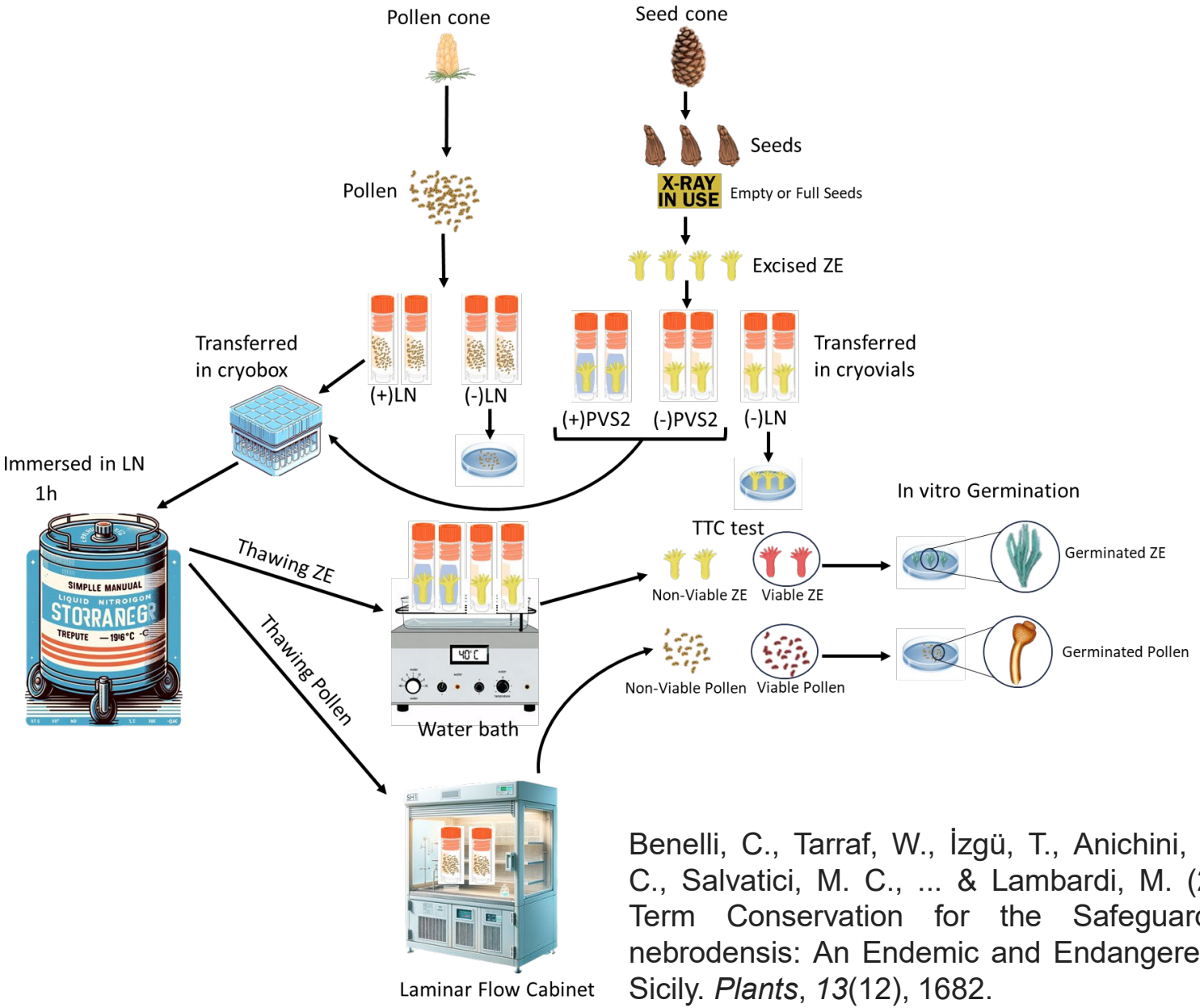
Full seed



Empty seed



Larva



Benelli, C., Tarraf, W., İzgü, T., Anichini, M., Faraloni, C., Salvatici, M. C., ... & Lambardi, M. (2024). Long-Term Conservation for the Safeguard of Abies nebrodensis: An Endemic and Endangered Species of Sicily. *Plants*, *13*(12), 1682.