CGIAR
SCIENCE FOR A FOOD-SECURE FUTURE

What is cryopreservation all about and how is it applied to clonal collections and beyond

Bart Panis

Alliance Bioversity & CIAT

ANNUAL GENEBANKS MEETING 2025

CGIAR GENEBANKS

CIP INTERNATIONAL POTATO CENTER

What is cryopreservation ?

Cryopreservation

Cryopreservation is a process where cells or whole tissues are **preserved** by cooling to low **sub-zero temperatures**, such as (typically) **−196 °C** (the boiling point of liquid nitrogen).

At these low temperatures, **any biological activity**, including the biochemical reactions that would lead to cell ageing (and cell death), is **effectively stopped**.

Practically: storage happens in **big Dewar flasks** filled with liquid nitrogen

Freezing induced injury

1/ **Effect of low (not always “freezing” temperatures)** (membrane stability, metabolism,……)

2/ **Mechanical effects of extracellular ice crystals** at cell surfaces (breaking of tissues, disconnection of cells)

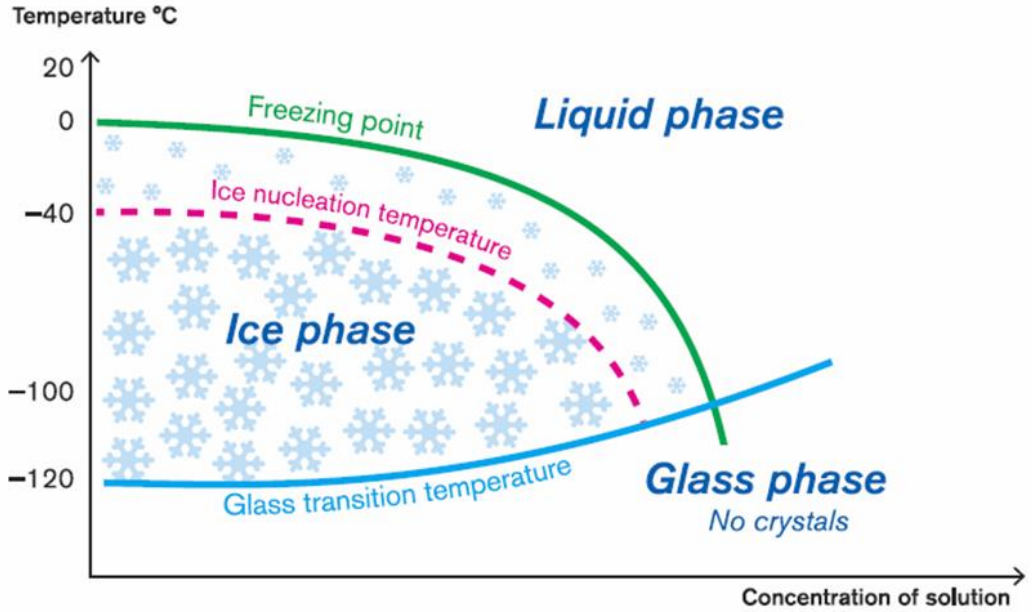
3/ **Dehydration related effects** (In nature, during cryopreservation when slow freezing rates are applied). Results in solution and mechanical effects

4/ Injury due to **intracellular ice formation**

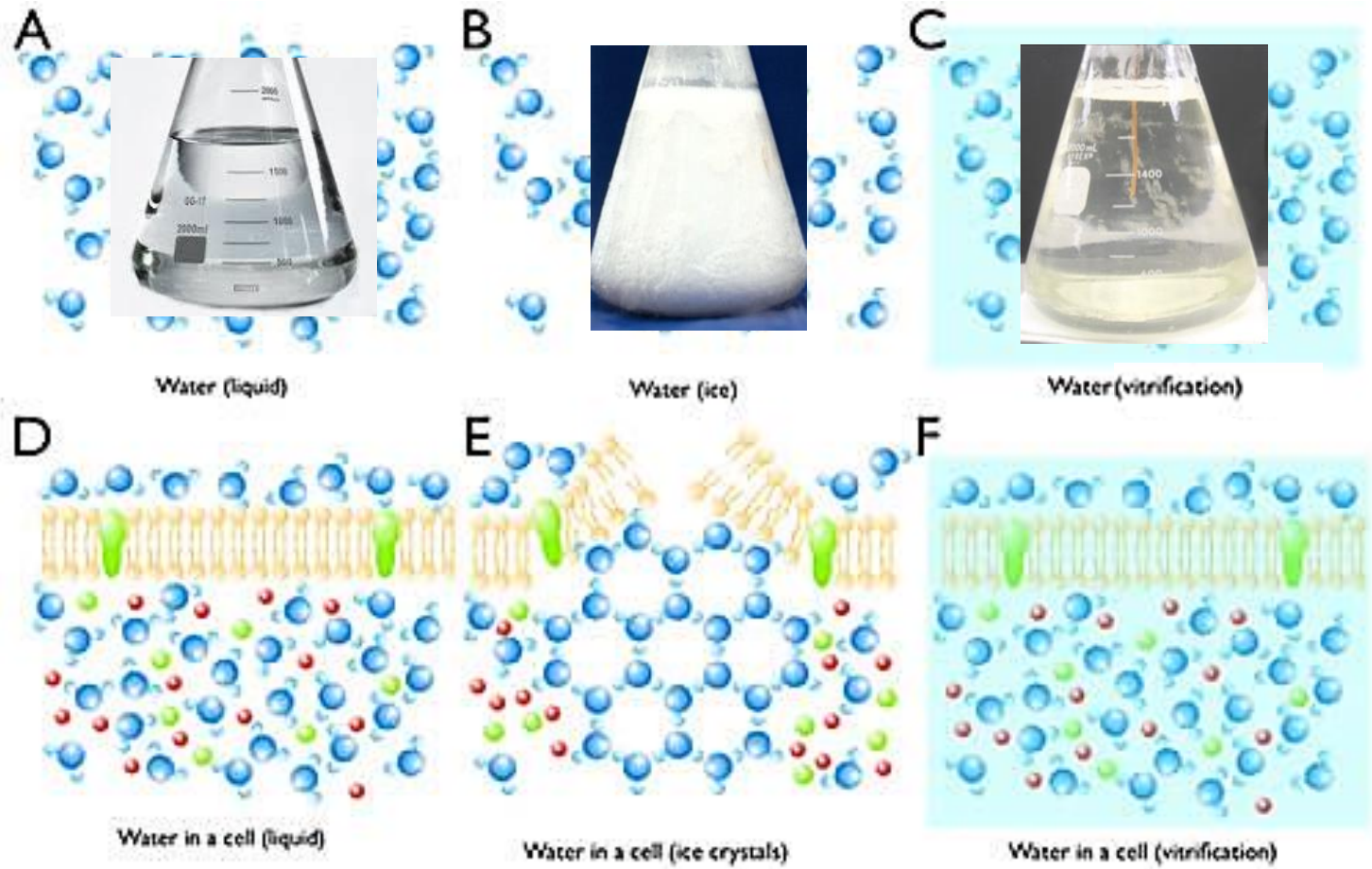
⇒ Mechanical disruption of protoplasmatic structure, loss of semi-permeability

Freezing induced injury

All cryogenic strategies rely on the prevention of intracellular ice crystal formation. The only way to prevent ice crystal formation at ultra-low temperatures without an extreme reduction of water content is through ‘vitrification’ (solidification of a solution without ice-crystals).



Vitrification

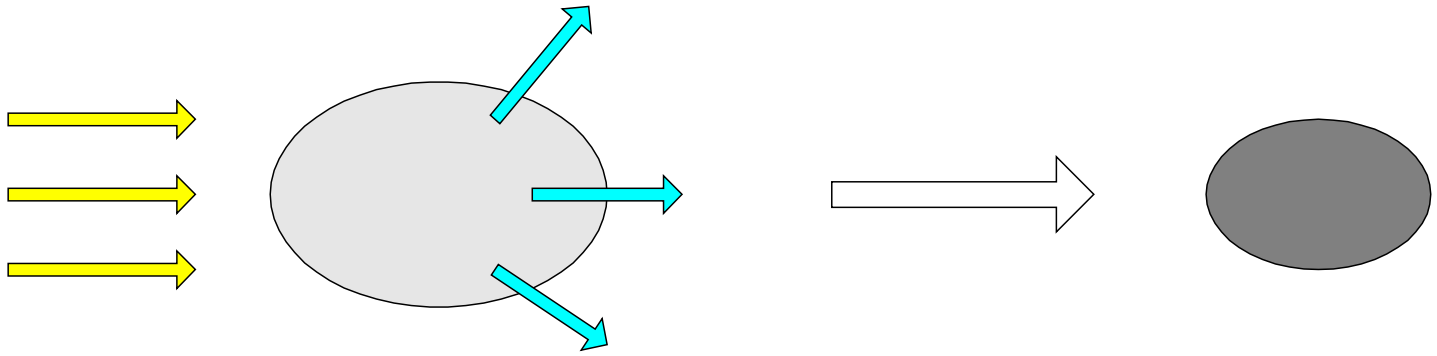


HOW???

1/ Concentration of cellular solution

2/ Rapid cooling and thawing rates

1. Concentration of cellular solution through air drying

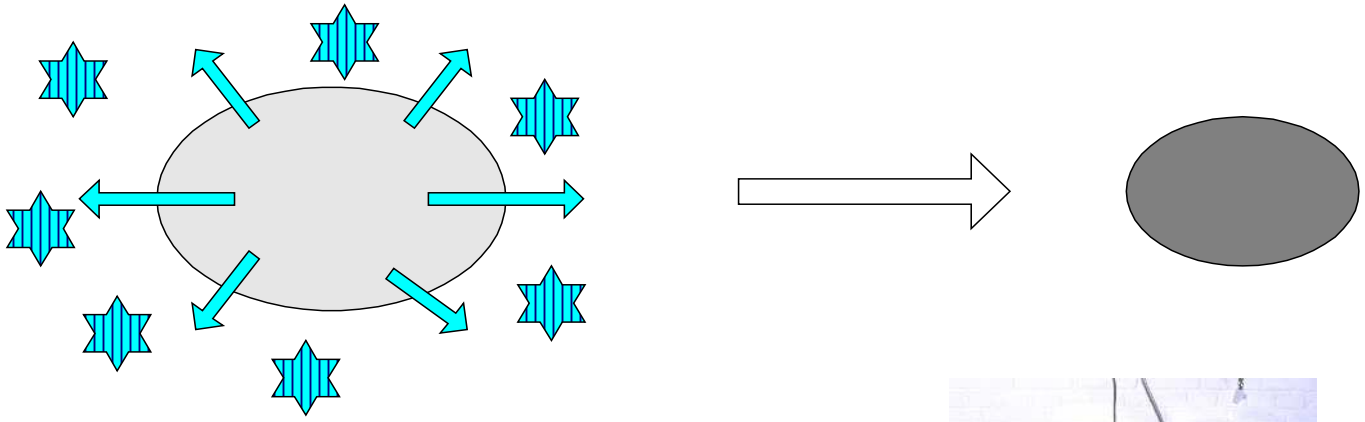


- Sterile air from laminar air flow cabinet

- Dry silica gel in a closed container

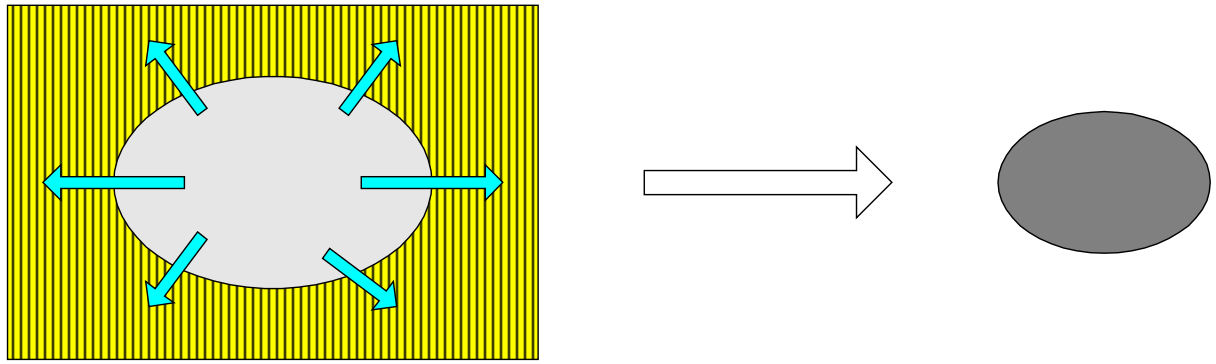
2. Concentration of cellular solution through freeze dehydration

Cooling rates : 0.3 to 10°C/min until -30 to - 50°C



- Computer driven cooling device
- stirred methanol bath
- propanol container (Mr Frosty)

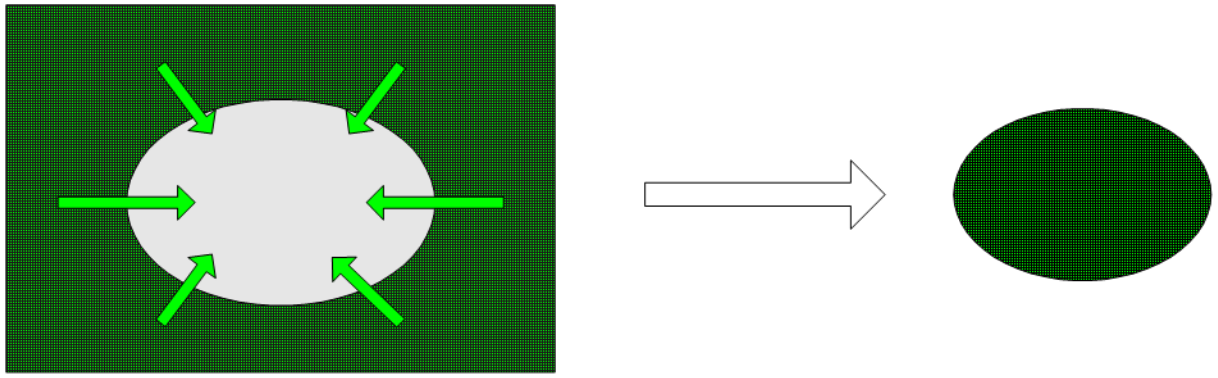
3. Concentration of cellular solution through osmotic dehydration



Non-penetrating cryoprotective substances

- Sugars
- Sugar alcohols
- High molecular weight additives (PEG,….)
- EG at low (0°C) temperature

4. Concentration of cellular solution through the addition of penetrating cryoprotective substances



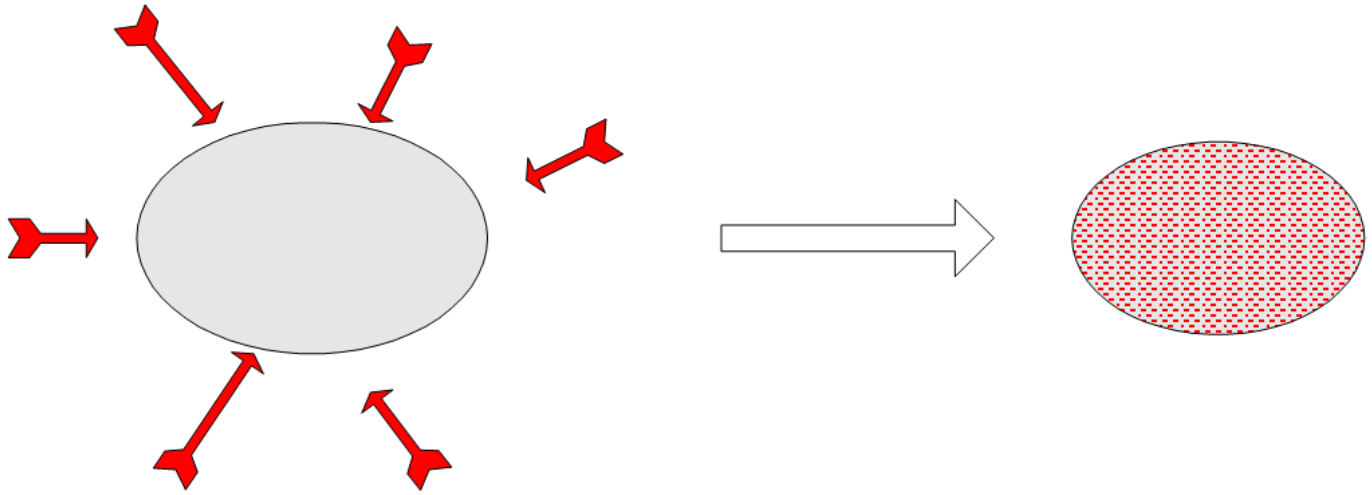
Colligative effect of penetrating cryoprotective substances

DMSO

Glycerol amino acids

EG at high temperature (RT)

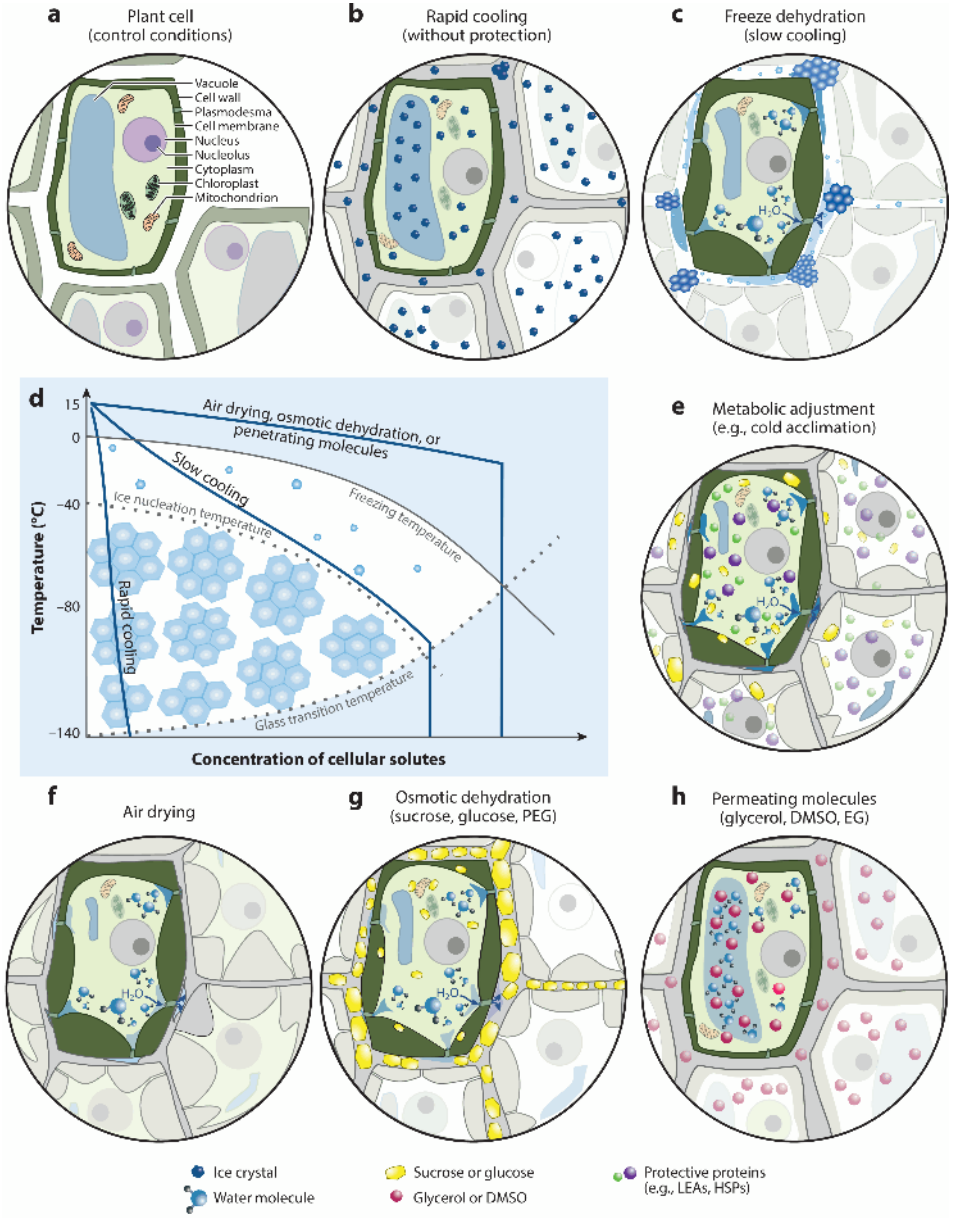
5. Concentration of cellular solution through adaptive metabolism



Induced by temperature changes, changes in light regime, osmotic changes, ABA,…..

Result : increase in proteins, sugars, glycerol, proline, polyamines, glycine betaine,... which have (among others, see later) colligative effects

Induction is genetically defined



Nagel M, et al. 2024
Annu. Rev. Plant Biol. 75:797–824

ANNUAL REVIEWS

Annual Review of Plant Biology

Plant Cryopreservation: Principles, Applications, and Challenges of Banking Plant Diversity at Ultralow Temperatures

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[5]Department of Cell Biology and Biotechnology, K.A. Timiryazev Institute of Plant Physiology, Russian Academy of Sciences, Moscow, Russia

[6]The Alliance of Bioversity International and the International Center for Tropical Agriculture (CIAT), KU Leuven, Leuven, Belgium

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- **Problem**: Most hydrated tissues do not withstand dehydration to moisture contents needed for vitrification (20-30 %). Exceptions are pollen, seed and somatic embryo of orthodox species.

- **The key for successful cryopreservation thus lies in the induction of tolerance towards dehydration.**

- **How ?**

  Non-colligative effects of

  1/ Addition of cryoprotective substances (Sugars, glycerol, DMSO,…) / Loading :

  2/ Adaptive metabolism (hardening):

Physico-biochemical parameters related to plant cryopreservation

CRYMCEPT
plant cryopreservation

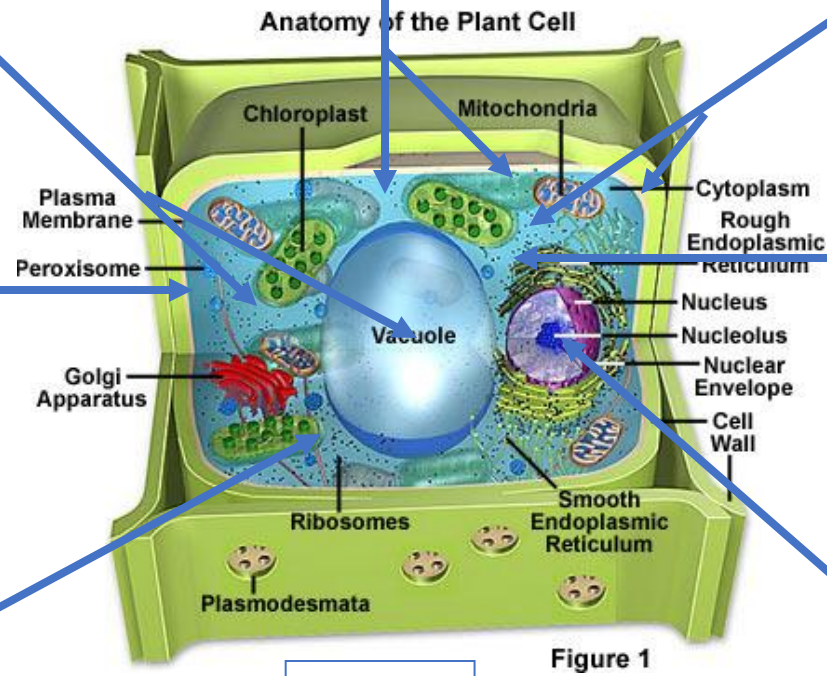
Water thermal behavior

Proteins

Sugars

Membrane components

Polyamines



Cytoskeletal protein

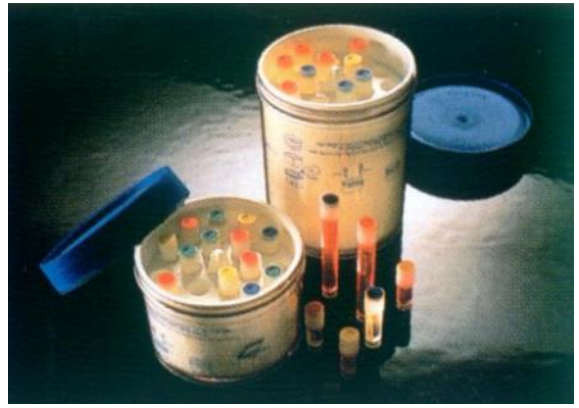
Oxidative stress

Plant cell

All cryopreservation protocols are combinations of 2 or more of cryogenic strategies

- Cold Hardening + Penetrating cryoprotective substances (Carnation shoots)
- Cold Hardening + Freeze dehydration (mulberry shoots)
- Cold Hardening + Air drying (mulberry shoots)
- Cold Hardening + Air drying + Freeze dehydration (apple shoots)
- Cold Hardening + 0smotic dehydration + Freeze dehydration (pear shoots)
- Cold Hardening + 0smotic dehydration + penetrating cryoprotective substances +Freeze dehydration (*Rubus* shoots)
- Cold Hardening + 0smotic dehydration + penetrating cryoprotective substances (apple shoots)
- Cold Hardening + Sugar Hardening + 0smotic dehydration + penetrating cryoprotective substances (mulberry shoots)
- Sugar Hardening + 0smotic dehydration + penetrating cryoprotective substances (mint shoots)
- Sugar Hardening + Air drying (oil palm somatic embryos)
- Sugar Hardening + osmotic dehydration + penetrating cryoprotective substances (Banana shoots)
- Penetrating cryoprotective substances + Freeze dehydration (Classical freezing method (banana cell suspensions))
- …….

Different cryopreservation protocols



?

Methods for cryopreservation

- Dormant bud cryopreservation
- Air drying
- Slow (classical) freezing
- Encapsulation-dehydration
- Droplet freezing
- Preculture + fast freeze (+ dehydration)
- Vitrification (PVS2 , PVS3,…)
- Encapsulation-vitrification
- Droplet vitrification
- V-cryo-plate procedure
- D-cryo plate procedure

Cryopreservation of dormant buds

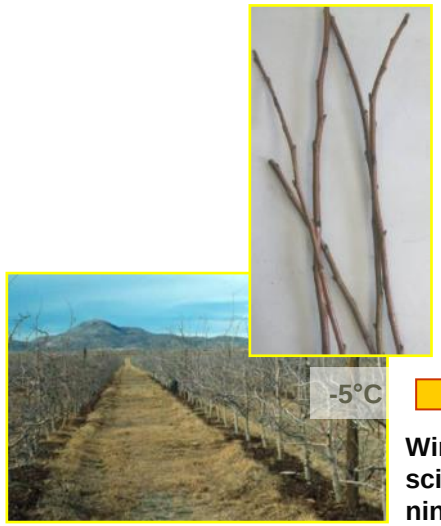
Akira Sakai (1956 dormant buds of Mulberry), (1960 dormant buds of Salix and Populus), Cecil Stushnoff, 1987 (dormant bud cryopreservation of apple)

Cold hardening (exposure to winter temperatures)
Air dehydration at -5°C to 25-35% MC
Freeze dehydration at -1°C/hr to -35°C – hold 24hr

Parameters to be optimised

- Hardening
- Cooling rate
- Holding temperature

Dormant bud cryopreservation (credits Maurizio Lambardi)



Winter collection of scions and cold harde ning at -5°C (8 wks)

Uni-nodal sections, cold hardened at - 4°C and dehydrated up to a MC of 35-26% (6-8 wks)

Slow cooling up to -30°C (-1°C/hour)

Thawing and rehydration in damp peat at 2°C (2 wks)

Chip budding

After 6 wks

After 1-2 wks

Based on Cecil Stushnoff, 1987

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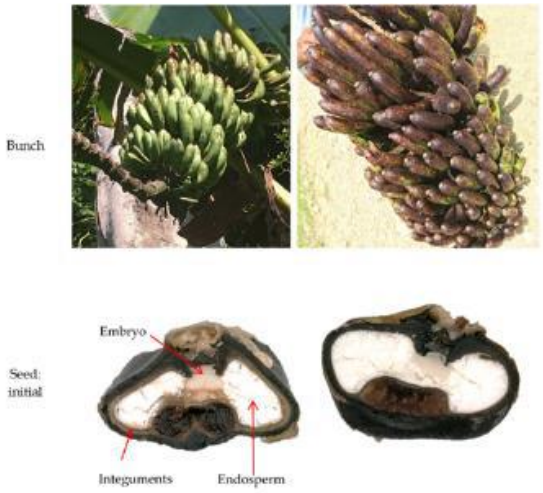
Air drying

Applied to orthodox seed, zygotic embryos and pollen



Wild Banana seed

☺ : Simple
No sophisticated equipment needed

☹ : Limited application (pollen, seed, zygotic embryos of orthodox seed)

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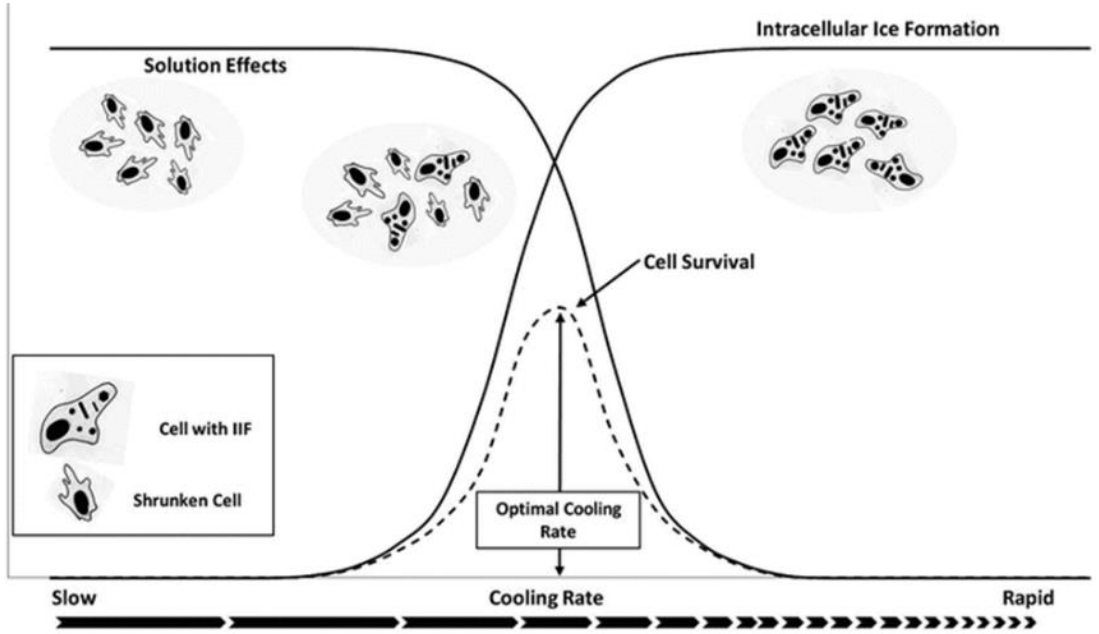
Classical (slow) freezing protocol

Nag and Street, 1973 ; Withers and King 1980; classical slow freezing (carrot cell suspension)

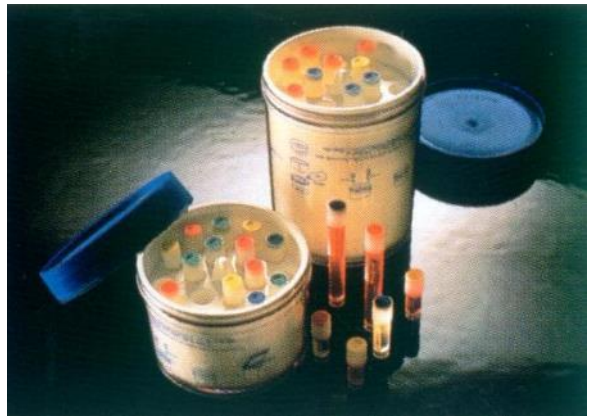
- Cold hardening and/ or Osmotic dehydration and/or Sugar hardening
- Penetrating cryoprotectants (often DMSO)+ non-penetrating cryoprotectants
- Freeze dehydration at 1°C /min to -35°C

<u>Parameters to be optimised</u>

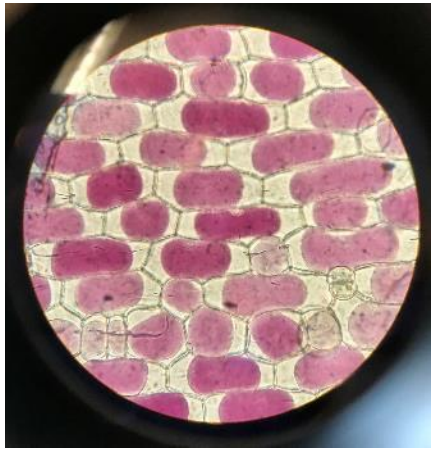
- Hardening
- Cryoprotective mixture (Often including DMSO)
- Cooling rate
- Holding temperature

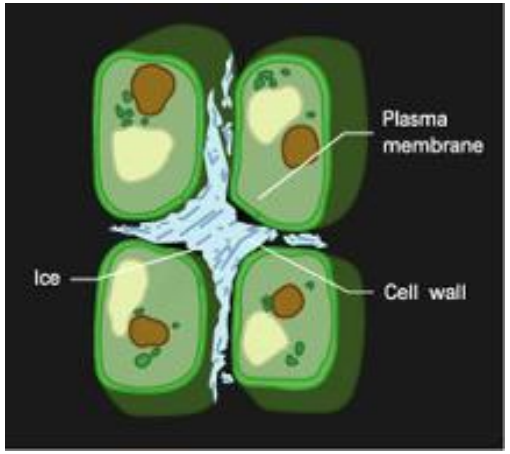

Intracellular Ice Formation
Solution Effects
Cell Survival
Cell with IIF
Shrunken Cell
Optimal Cooling Rate
Slow
Cooling Rate
Rapid


cryocool
cryotainer



Problem Plasmolysis Extracellular ice





☺ : Applicable to cell suspensions and callus (unorganised tissues)

☹ : More limited application to organised tissues (meristems cultures)
Expensive cooling devices are sometimes needed

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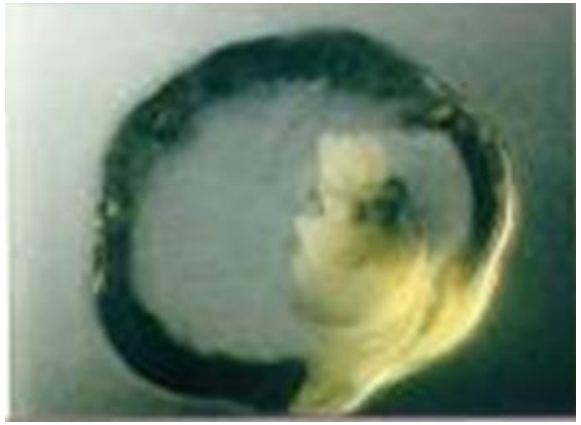
Encapsulation/dehydration

Fabre and Dereuddre, 1990 (encapsulation –dehydration of pear)



Typical protocol

- Encapsulation in alginate beads
- Treatment with high sucrose (1-2 M) for 1-5 days
- Air Drying (to 20-30 % moisture content)

(Cold hardening) + Sugar Hardening + 0smotic dehydration + Air drying

Parameters to be optimised :

- Hardening
- Sugar treatment (concentration, length, regime)
- Air drying

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Vitrification

Sakai et al., 1990 (PVS2 vitrification nucellar cells of navel orange)

Typical protocol

- Loading : LS : 2 M glycerol + 0.4 M sucrose
- Dehydration : PVS2 : 30 % glycerol + 15 % EG + 15 % DMSO + 0.4 M sucrose
- Following freezing and thawing : deloading in 1.2 M sucrose

(Cold Hardening) Sugar hardening + osmotic dehydration + penetrating cryoprotectants (at 0°C or RT)

Parameters to be optimised

- Sugar hardening
- Loading
- Dehydration with vitrification solution (temp, time, composition,…)

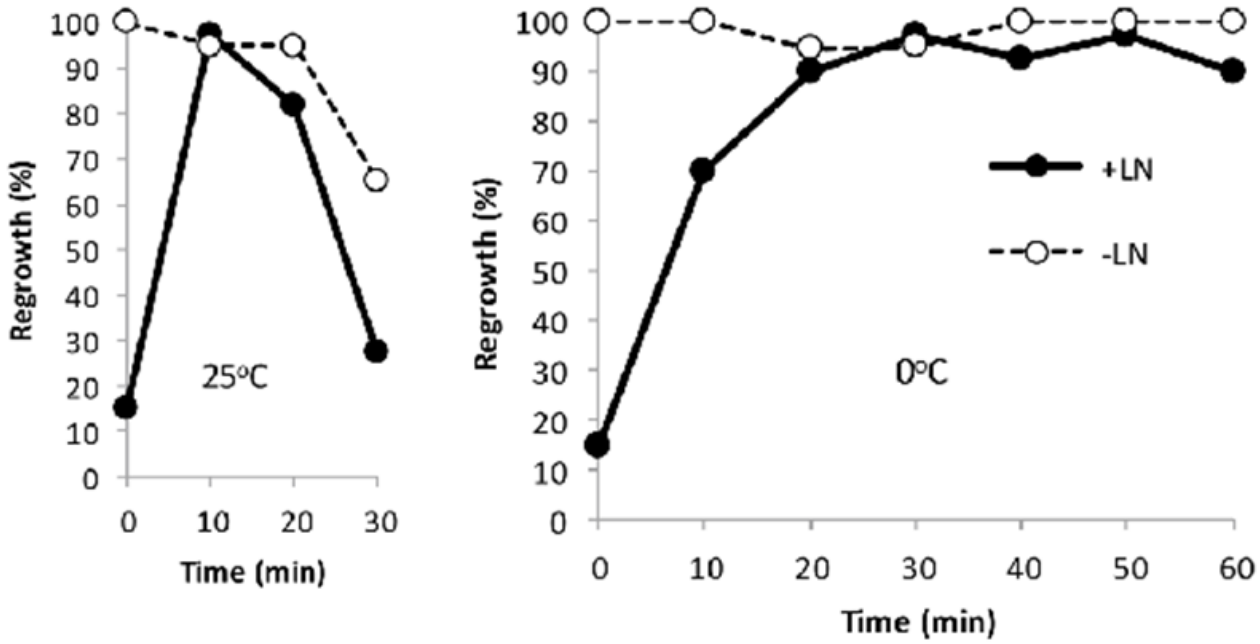

Fig.1. Effect of exposure time to PVS2 at 25 or 0 °C on recovery growth from wasabi shoot tips cooled to -196 °C by vitrification. Shoot tips (1 mm size) were precultured with 0.3 M sucrose for 1 d and then treated with a mixture of 2 M glycerol plus 0.4 M sucrose (LS solution) for 20 min at 25 °C. These shoot tips were treated with PVS2 for different lengths of time prior to immersion in LN. (Matsumoto *et al.*, 1994).

☺ : Protocol applied to a wide range of culture types and plant species
No slow cooling devices are needed

☹ : Susceptibility to 'toxic' Vitrification solution is species dependent
rather time consuming and labour intensive protocol

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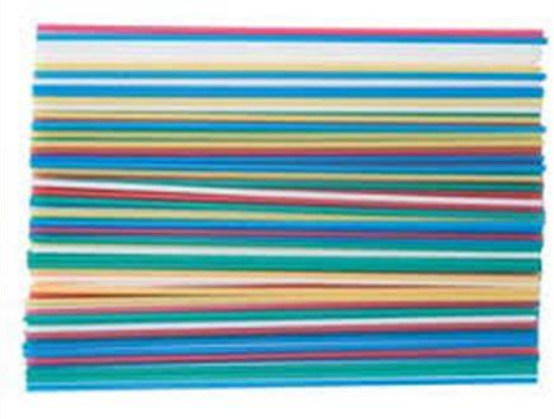
Droplet-vitrification

Towill and Jarret, 1992 (First “droplet vitrification” on sweet potato)

Combination of the Classical vitrification (with PVS2 or PVS3 or….) and the application of ultra fast freezing and ultra fast warming (to avoid respectively crystallization and cold crystallization).

HOW? A closer contact between the tissue and the cooling agent.

- Cryotubes (about 6°C/sec)
- Semen straws (about 60°C/sec)(potato)
- Droplet vitrification (about 130°C/sec)

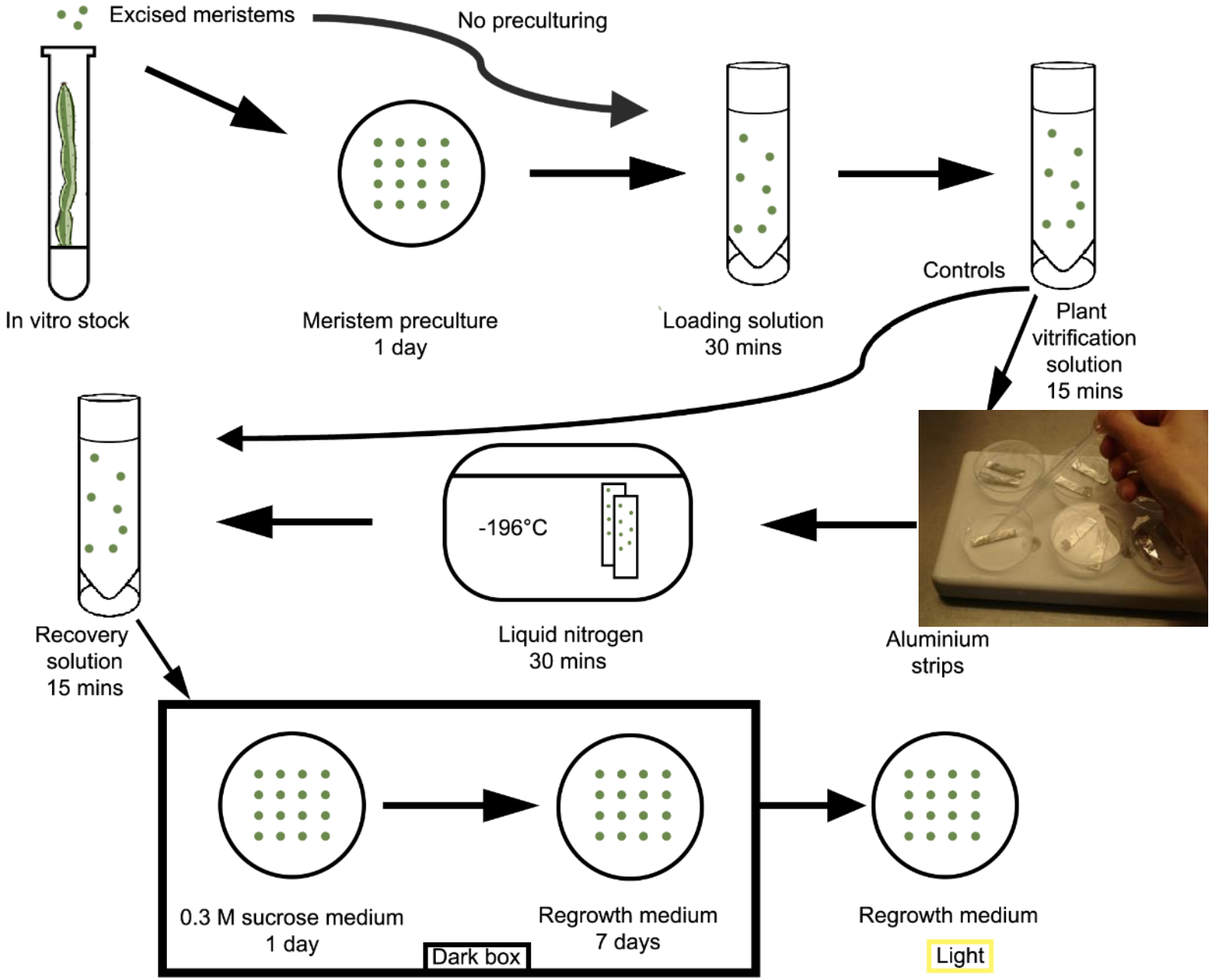


Droplet vitrification

Survival

≠

Regeneration



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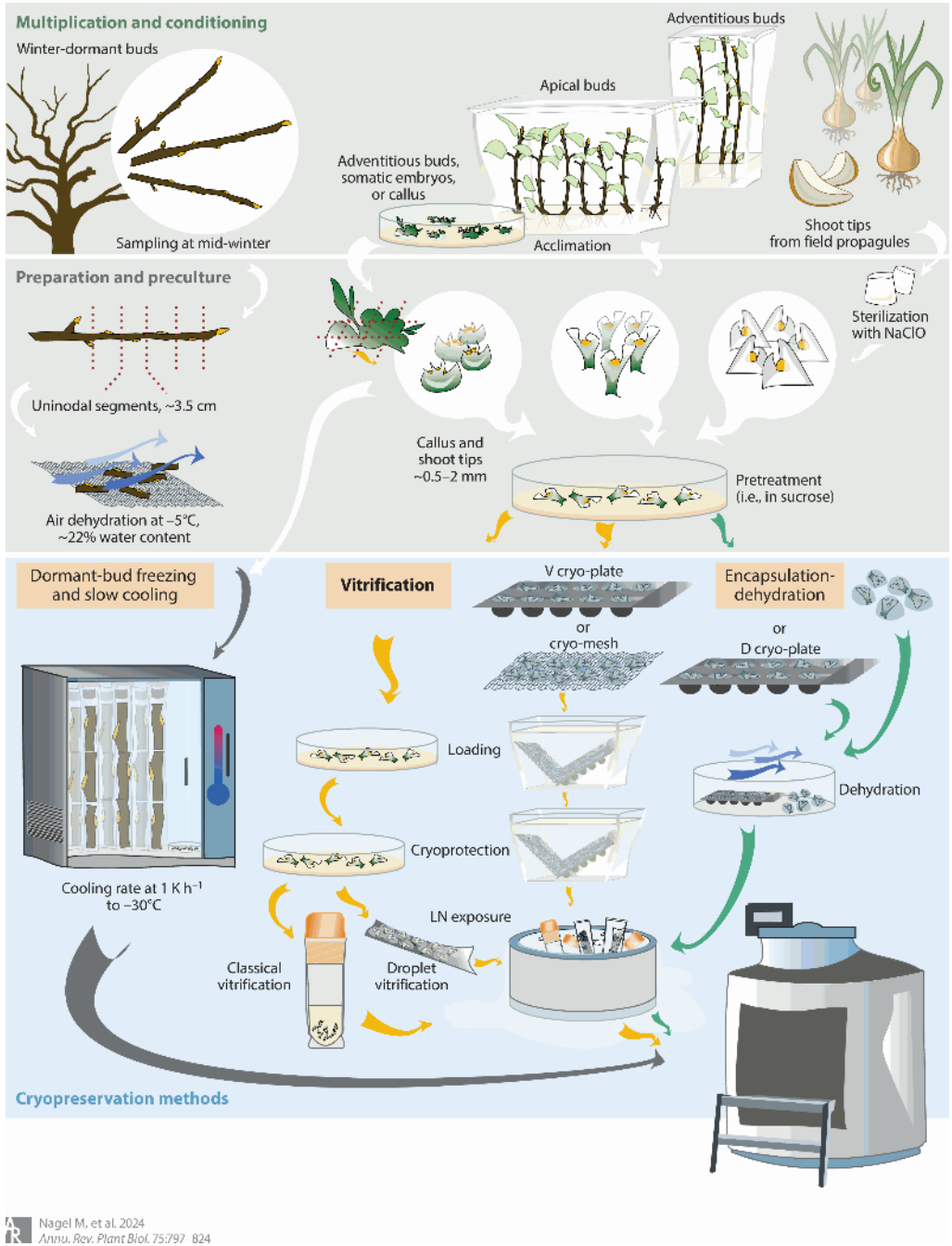

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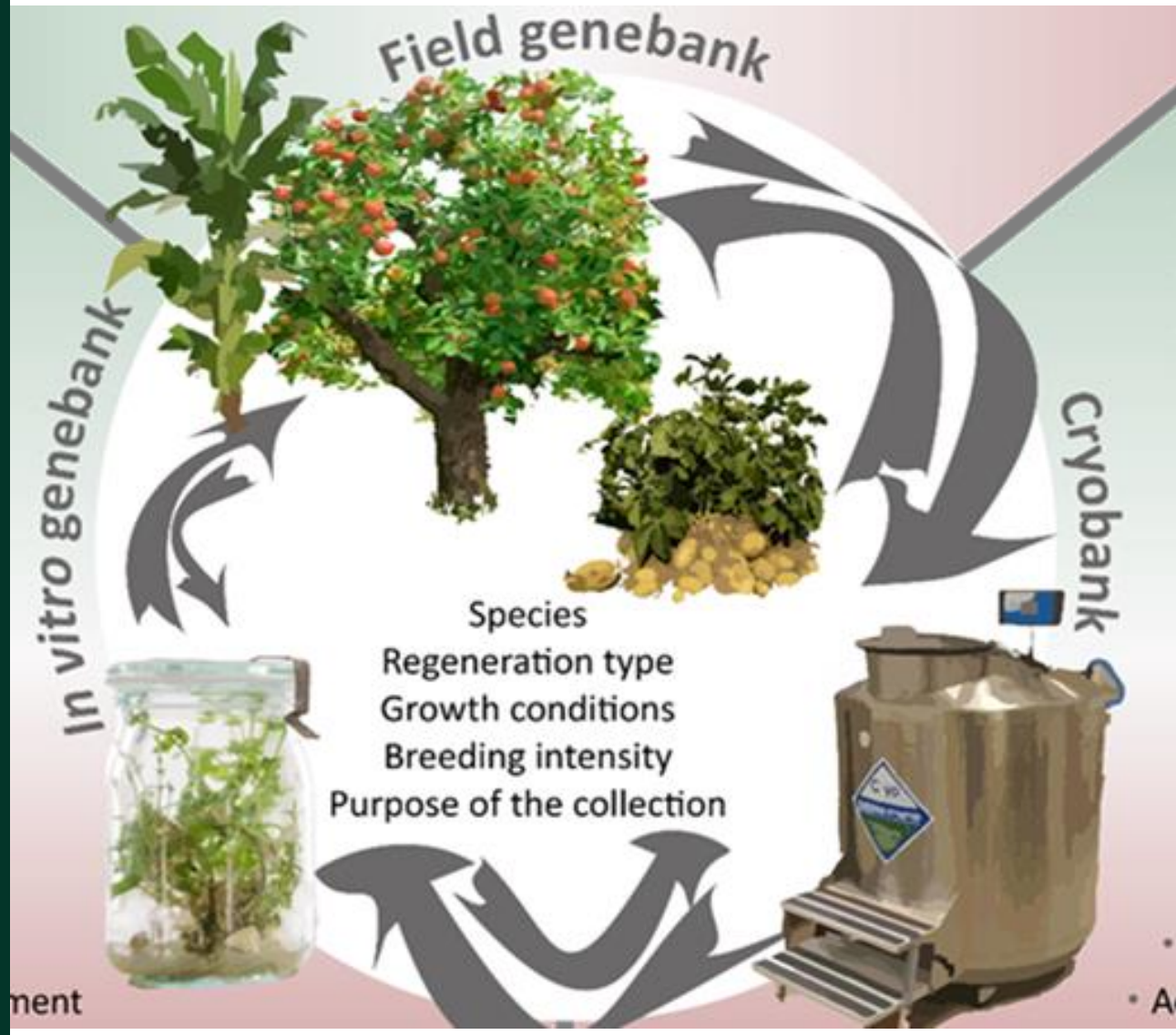
Institute	Crop	Cryopreservation Method
Bioversity International, Leuven, Belgium	Banana	• Droplet vitrification
Association FOrêt-CELlulose (AFOCEL), France	Elm	• Dormant bud freezing
International Center for Tropical Agriculture (CIAT), Cali, Colombia	cassava	• Droplet vitrification • Encapsulation/dehydration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

Cryopreservation for storage of genetic resources



Methods of conservation

- *In situ* : Conservation in ‘normal’ habitat
  - rain forests, gardens, farms
- *Ex Situ* :
  - **Seed collections**
  - Field collection, Botanical gardens
  - In vitro collection
    - Normal growth
    - Slow growth (temp↘, O_2 ↘, H_2O ↘, medium ~)
  - Cryopreservation (-196°C)
- (DNA Banks)

CIAT Bean genebank, Colombia

Svalbard Global Seed Vault

> 1 million seed samples

Many Critical Food and Nutrition Security Crops Cannot be Conserved in Perpetuity by Seeds

- Seedless crops
- Crops that do not breed true from seeds
- Crops with recalcitrant or short-lived seeds

Solution :

- cryopreservation of seed or embryos
- Store vegetative tissues

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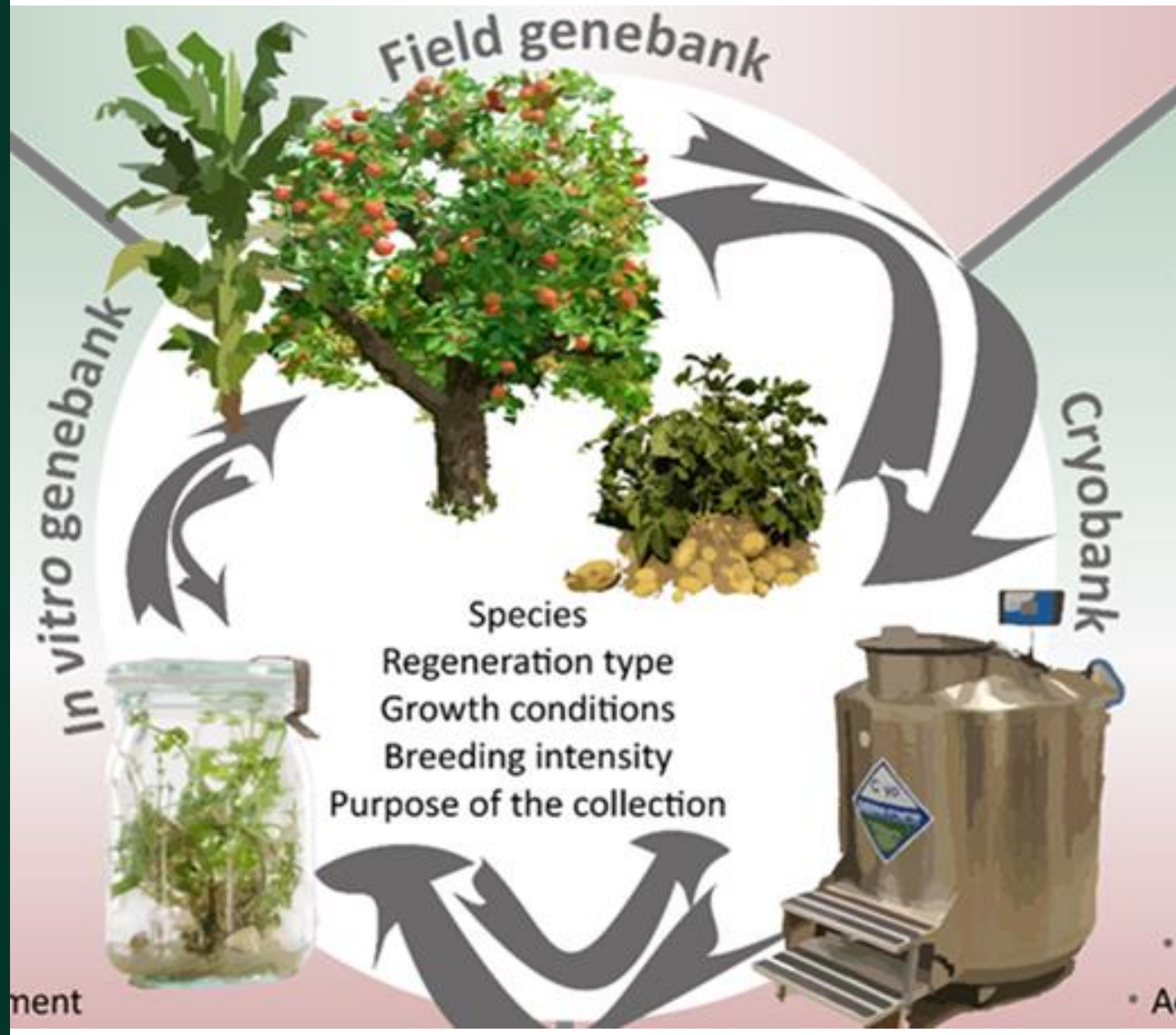
- *Ex Situ* :
  - Seed collections
  - Field collection, Botanical gardens
  - In vitro collection
    - Normal growth
    - Slow growth (temp↘, O_2 ↘, H_2O ↘, medium ~)
  - Cryopreservation (-196°C)
- (DNA Banks)

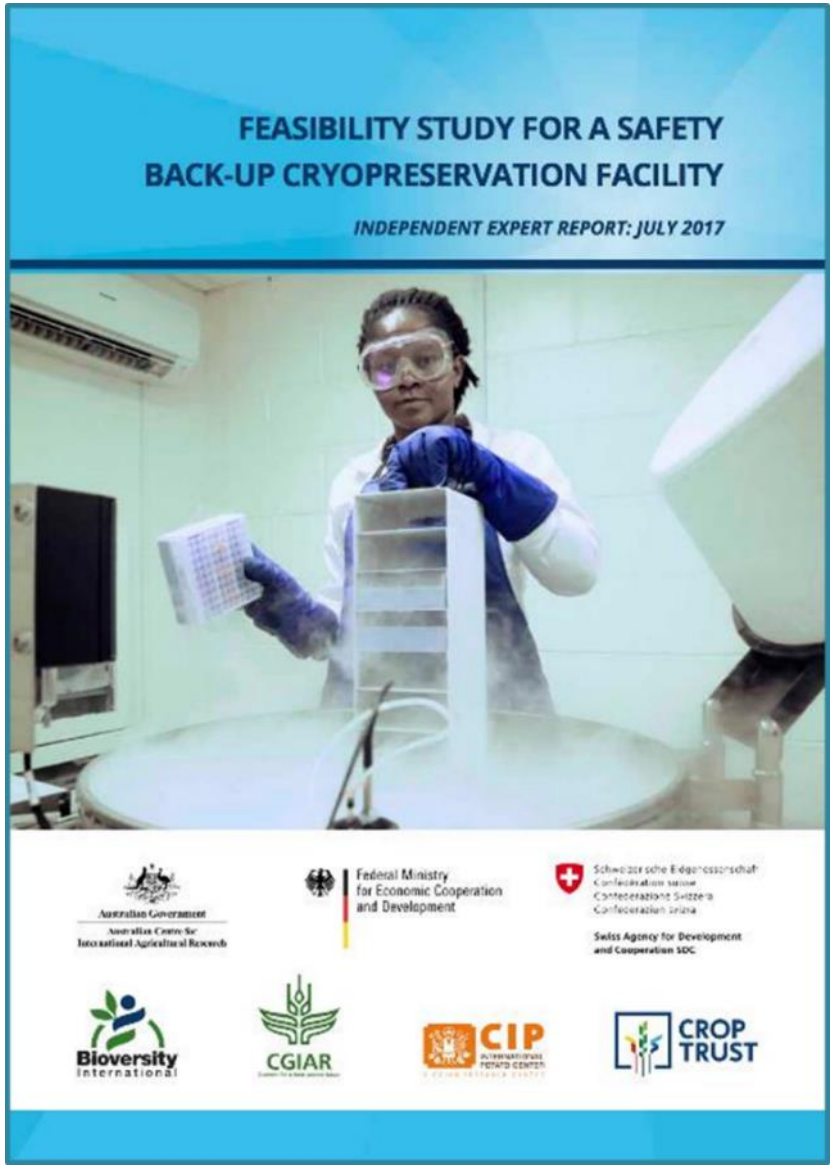
IPK potato collection, Germany

Bioversity International in vitro banana collection, Belgium

Bioversity International Cryobank, Belgium

Cryopreserved collections





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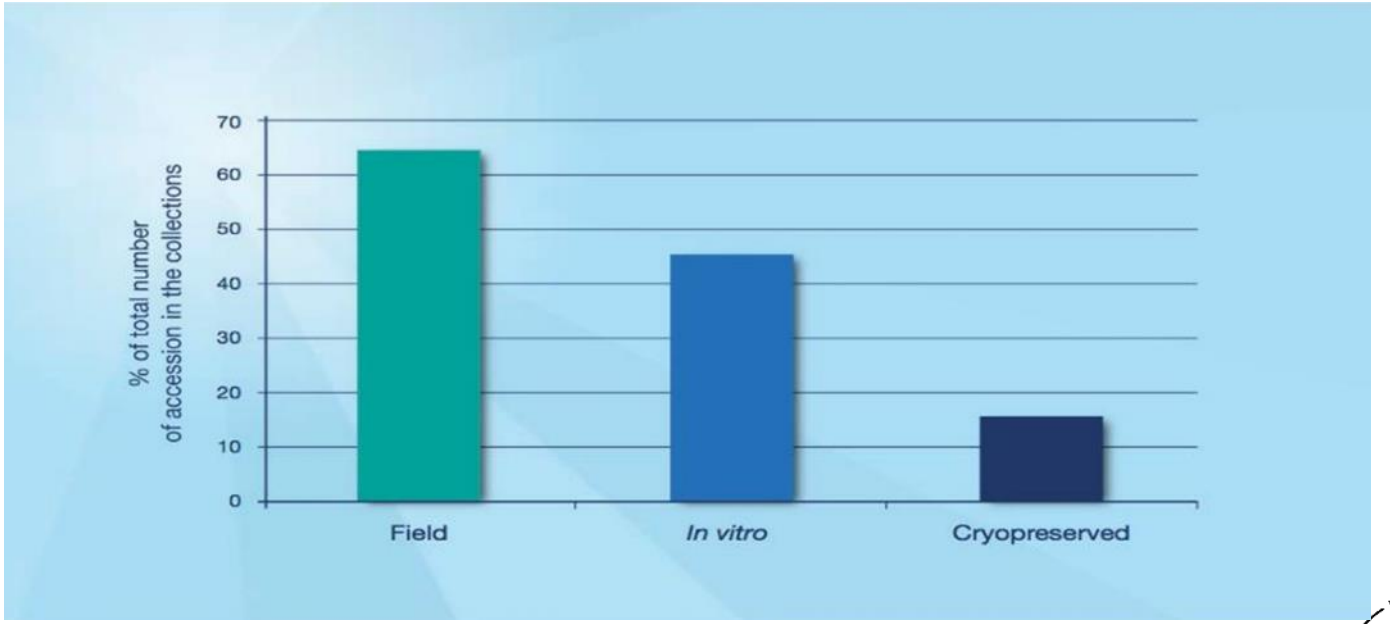
Survey was sent to 26 organizations around the world holding existing or emerging cryo-collections, 19 responded

Status of cryopreserved crop collections

- 15 institutes together hold 9,650 accessions of 30 crops in cryopreservation
- Only 9 crops have cryopreserved collections of more than 100 accessions !
- This constitutes only 16% of the total number of accessions they collectively hold of these crops.
- The majority of the accessions are maintained in the field (66%) and/or *in vitro* culture (46%).

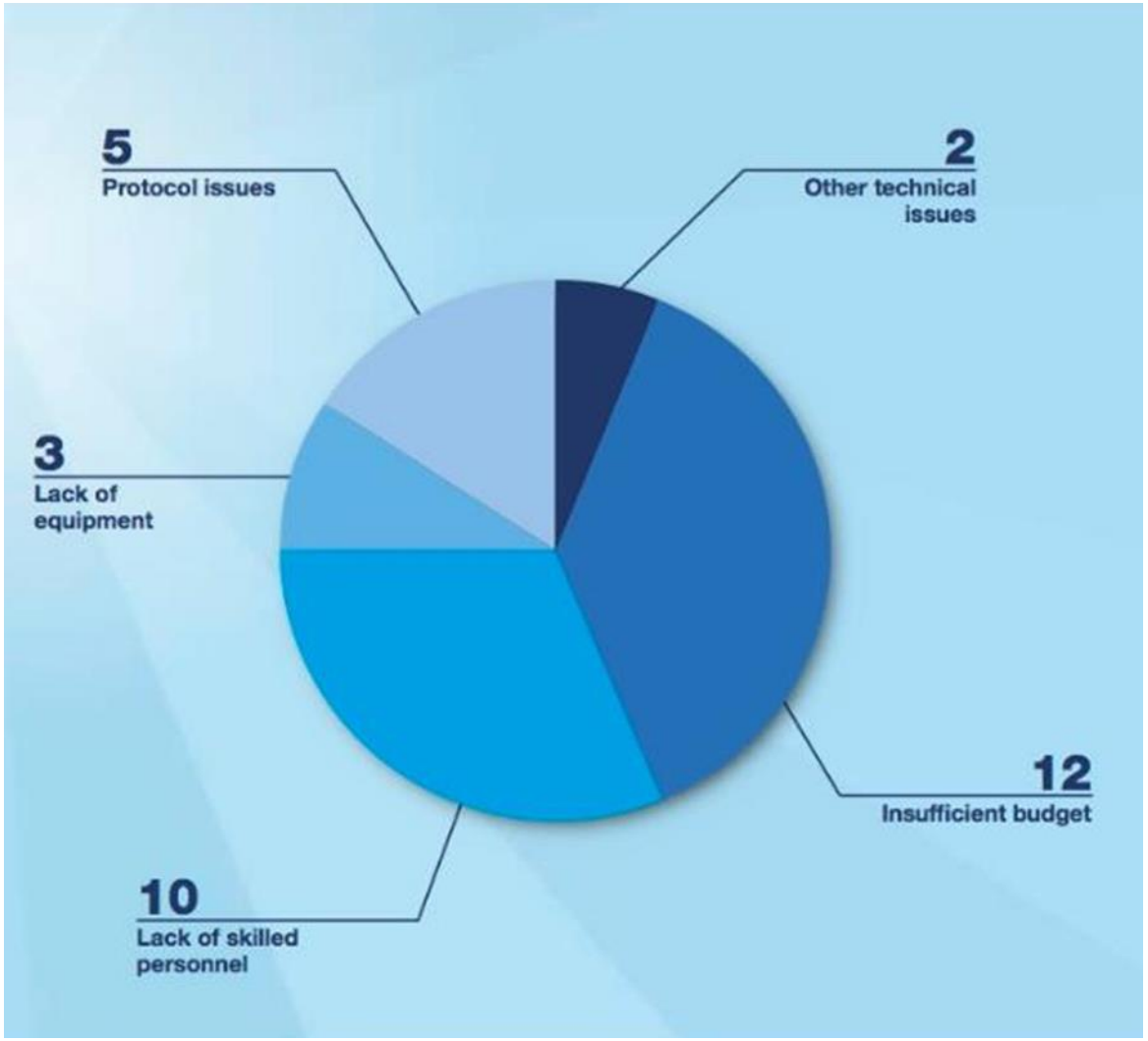


Major difficulties and constrains

- Challenges in **protocol development** (the science and methodology of cryopreservation)

- Challenges with the **implementation** of existing cryopreservation protocols (the genotype-specific issues in adapting the protocols to multiple accessions; effective work organization; sufficient supply of plant material, etc.)

- Challenges related to **cryobanking capacity** (insufficient funding, lack of skilled personnel, lack of equipment/infrastructure, etc.)



Results: How many accessions of clonal/recalcitrant seed crops are there?

Crop	Crop type	No. accessions in *ex situ* collections	Major holding institutes
Breadfruit and relatives	Clonal	3 158	SPC, Fiji USDA, USA NTBG, Hawaii, USA
Coffee	Recalcitrant/Intermediate seeds	30 483	CIRAD, France IAC, Brazil CATIE, Costa Rica
Coconut	Recalcitrant/Intermediate seeds	1 680	CPCRI, India PCA, Philippines IPRI, Indonesia
Major aroids	Clonal	7 394	SATRC, Papua New Guinea CPCT, Fiji MARDI, Malaysia
Cacao	Recalcitrant/Intermediate seeds	23 107	INIAP, Ecuador MCB, Malaysia CRU/UW, Trinidad and Tobago
Strawberry	Clonal	12 027	USDA, USA PGRC, Canada VIR, Russia
Cassava	Clonal	36 529	CIAT, Colombia Embrapa, Brazil IITA, Nigeria
Citrus	Clonal	36 410	CCSM-IASP, Brazil NIAS, Japan CRI, China

Results: How many accessions of clonal/recalcitrant seed crops are there?

Crop	Crop type	No. accessions in *ex situ* collections	Major holding institutes
Sweet potato	Clonal	35 478	CIP, Peru NIAS, Japan USDA, USA
Yam	Clonal	15 903	IITA, Nigeria PGRR, Ghana UNCI, Côte d'Ivoire
Banana and plantain	Clonal	11 606	ITC, Belgium CARBAP, Cameroon BPI, Philippines
Potato	Clonal/seeds	98 285	INRA, France VIR, Russia CIP, Peru
Taro	Clonal	7 302	WLMP, Papua New Guinea RGC, Fiji MARDI, Malaysia
Garlic	Clonal	29 898	NRCOG, India VIR, Russia NIAS, Japan
Tea	Intermediate seeds	11 838	VINATRI, Vietnam NIAS, Japan TARI, Taiwan
Apple	Clonal	59 922	GEN, USA VIR, Russia NIAS, Japan

GRAND TOTAL: **421 020 accessions** (including potential duplicates)

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Recommendations of the feasibility study ?

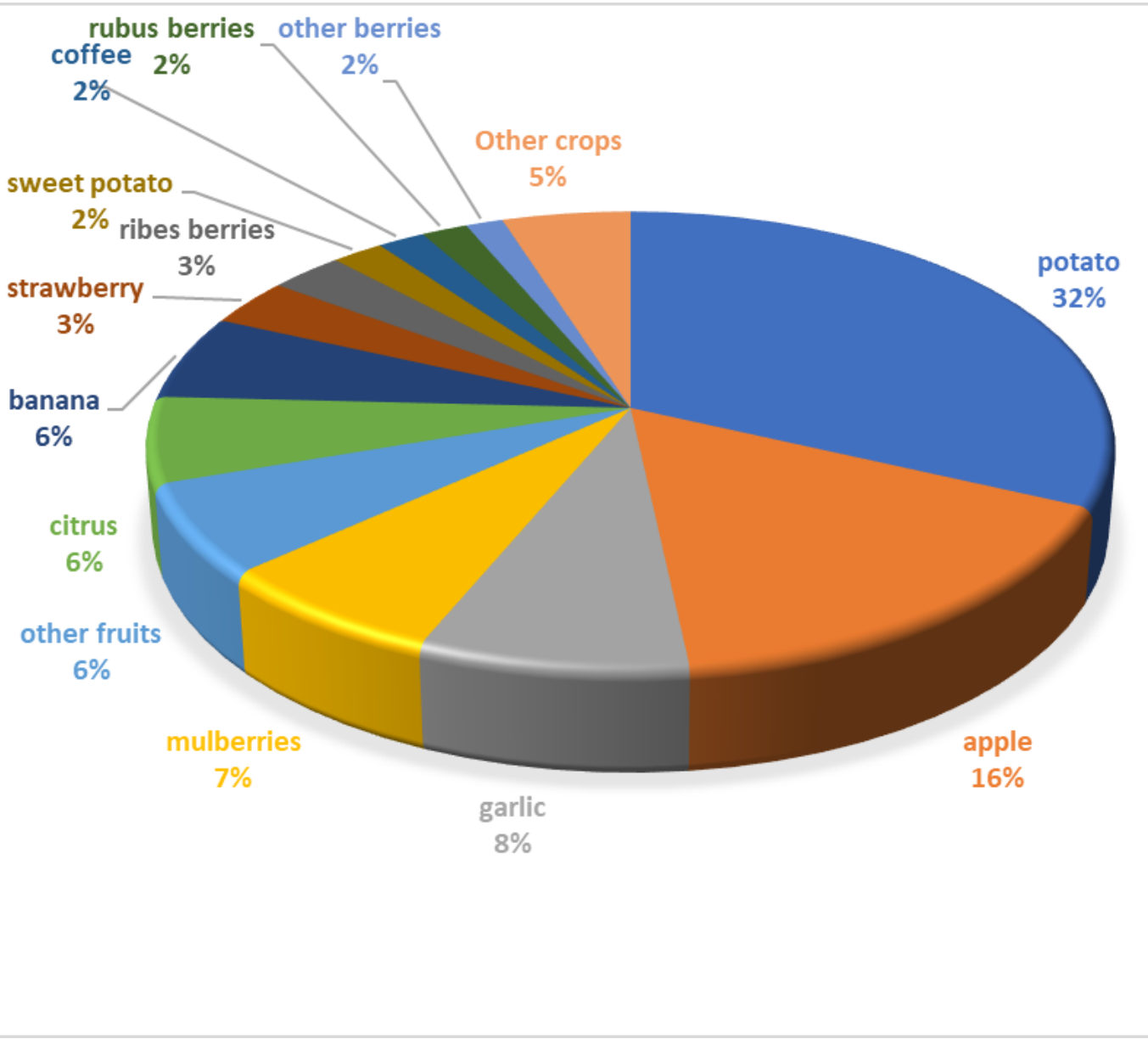
1. A major global initiative should be launched to accelerate the development and implementation of crop cryopreservation for important crops (estimate that 100 000 accessions need to be cryopreserved)

2. A back up cryopreservation facility should be set up to accommodate the estimated 5,000 to 10,000 accessions that are already cryopreserved (cfr Svalbard Seed Vault)

New survey in 2022

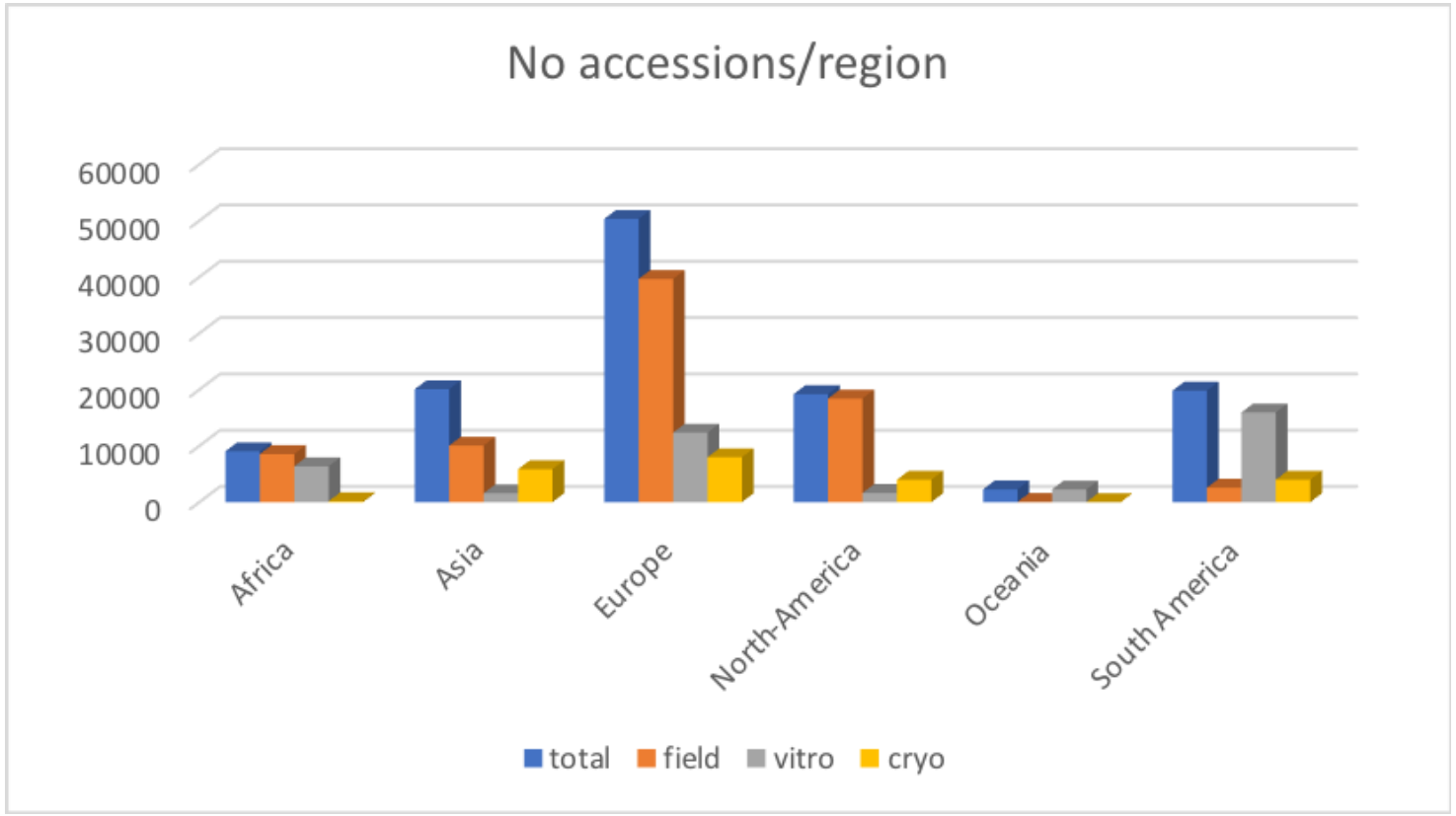
Year of Survey	Surveys sent	Data received	% Responses
2017	26	19	**73**
2021	29	27	**93**

New survey in **2022: 27** responded
17 crops have cryopreserved collections of more than 100 accessions !

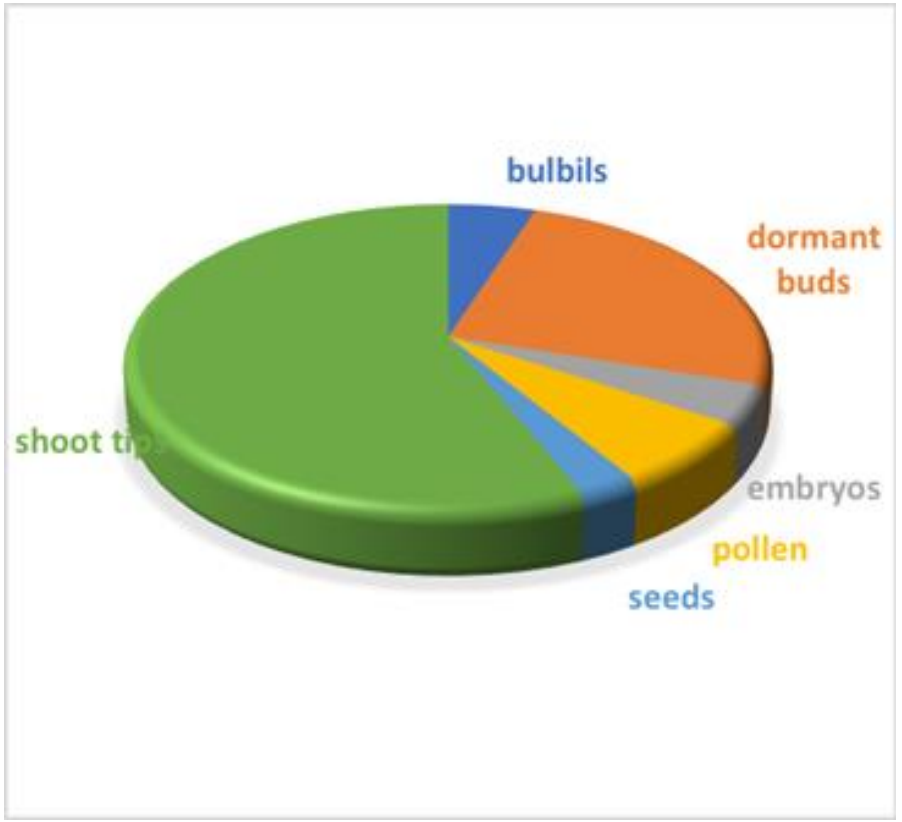


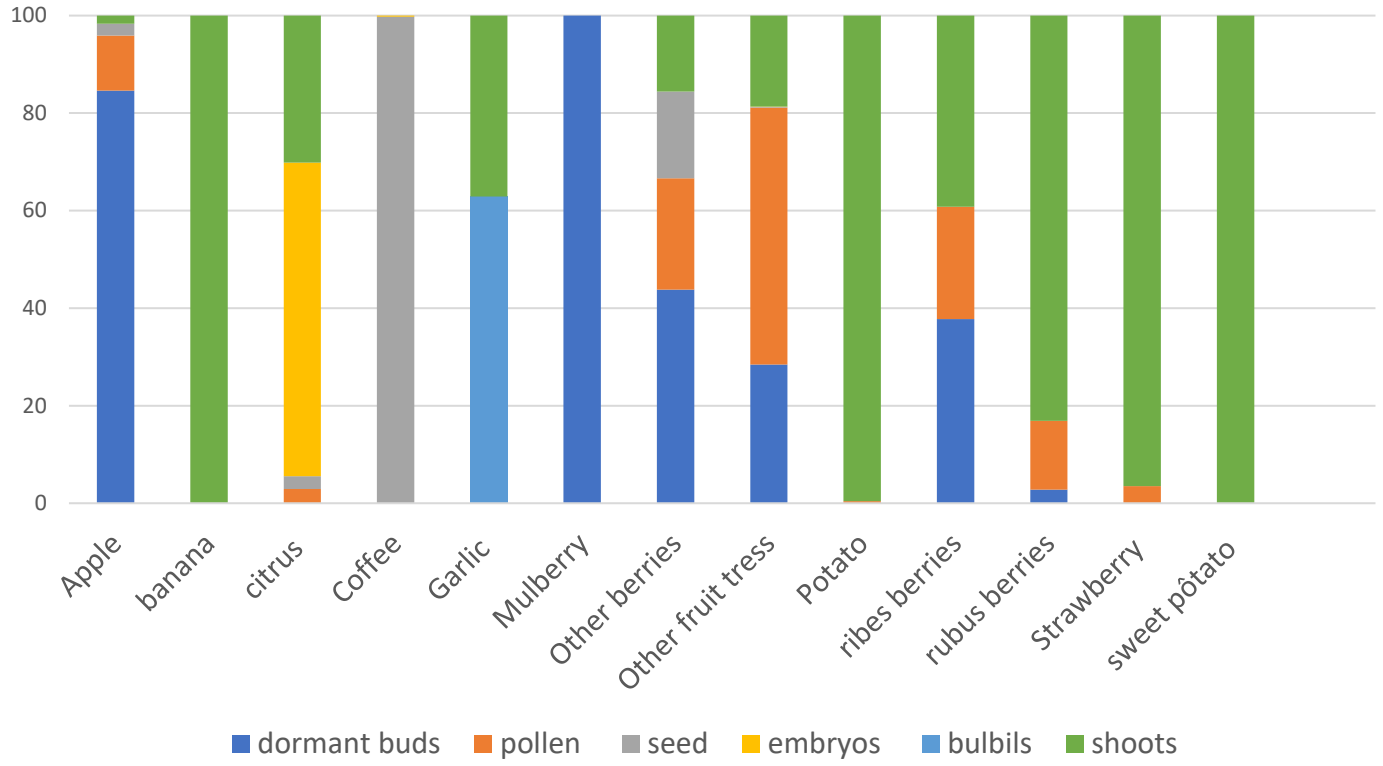
23 000 accessions cryopreserved (10 000 in the 2017 survey)

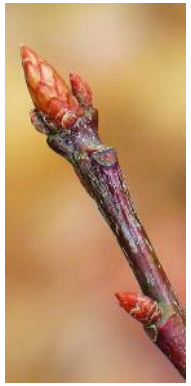
Geographic regions

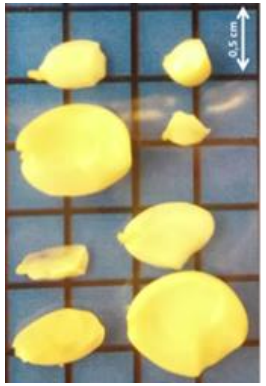


Regions with highest genetic diversity (often in the south) are less involved in cryo, their collections are more vulnerable to sudden threats such as COVID pandemic


bulbils
dormant buds
shoot tip
embryos
pollen
seeds


100
80
60
40
20
0
Apple
banana
citrus
Coffee
Garlic
Mulberry
Other berries
Other fruit tress
Potato
ribes berries
rubus berries
Strawberry
sweet pôtato
dormant buds
pollen
seed
embryos
bulbils
shoots




0.5 cm

The ITC (The banana genebank)

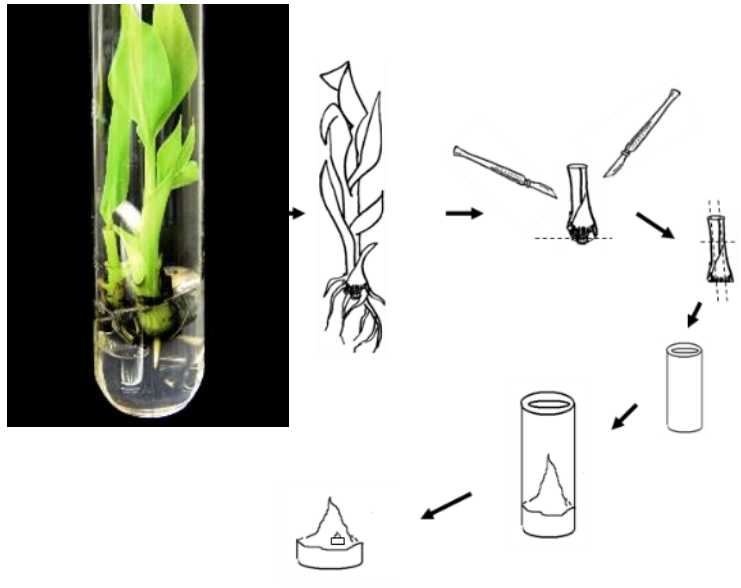
- Established in 1985 at KU Leuven, Belgium
- Food and Agriculture Organization of the UN ‘in trust’ collection (1994)
- Shared through the Multilateral System of Access and Benefit Sharing of the International Treaty on Plant Genetic Resources for Food and Agriculture (2004)

Cryopreserved base collection 1328 accessions

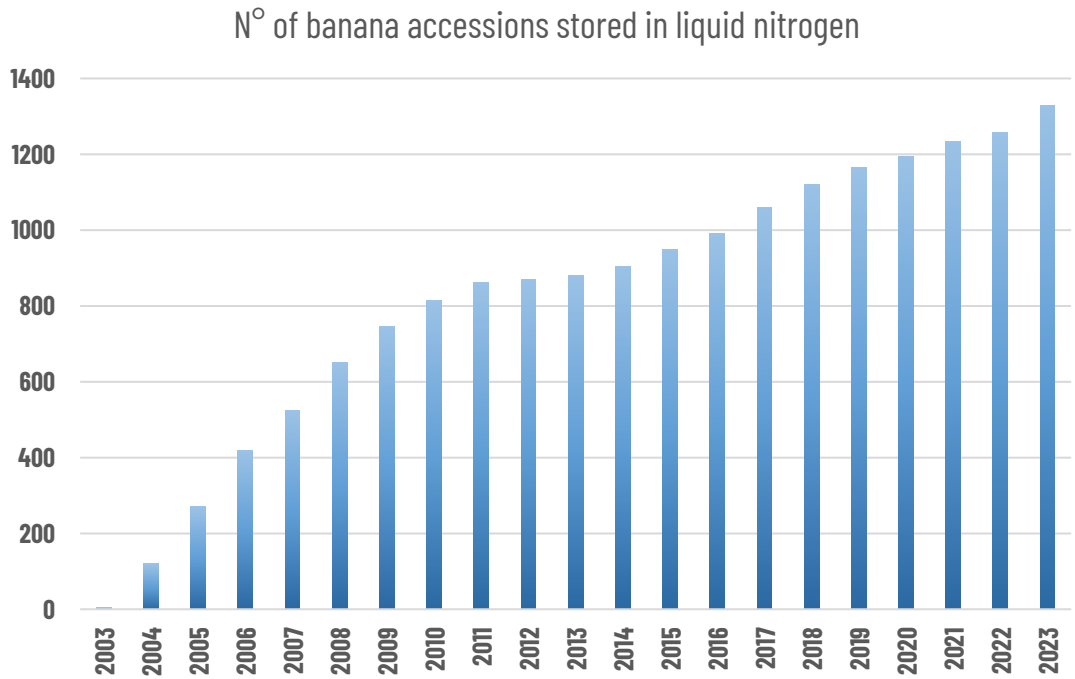
In vitro active collection 1716 accessions

Lyophilized leaf tissue collection 939 accessions

Off site black box safety back-up (IRD, France) 1181 accessions



Number of banana accessions cryopreserved



N° of accessions cryopreserved in the framework of

- World Bank : 200
- Gatsby foundation : 150
- BTC/Bioversity International : 100
- World Bank (GPG2) : 250
- TRUST, Gates foundation : 250
- Genebanking: 350…….

Major bottlenecks for banana cryo

- Optimized protocol is available
- Funds (40 accessions per person per year)
- Scale
- Availability of cryopreservable materials (virus free)

Equipment










Storage tanks, oxygen meters, level indicators, laminar flow benches, autoclaves, culture rooms, dry shipper, vacuum piping

Development of Cryopreservation protocols

- **Potato** (CIP, Peru; CRPGL, Luxemburg; VIR, Russia)
- **Ulluco** (CIP, Peru)
- **Sweet potato** (CIP, Peru)
- **Chicory** (CRA, Gembloux, Belgium)
- **Strawberry** (CRA, Gembloux, Belgium)
- **Taro** (SPC, Fiji)
- **Pelargonium** (INH, Angers, France)
- **Date Palm** (Université de Sfax, Tunesia)
- **Banana** (CIRAD, France; NBPGR, India)
- **Thyme** (Univ Alicante, Spain)
- **Olive** (IFAPA, Malaga, Spain)
- **Hop** (Univ Oviedo, Spain)
- **Photinia** (CNR, Firenze, Italy; Gebze, Turkey)
- **Vitis** (CNR, Palermo, Italy, PFR, Palmerston, NZ)
- **Apple** (Fruit Tree Research Institute, Italy)
- **Cassava** (IITA, Nigeria, CIAT, Colombia)
- **Tomato** (University of Valencia, Spain)
- **Bituminaria** (University of Valencia, Spain)
- **Narcissus** (Daffodil) (University of Krakow, Poland)
- **Galanthus** (Snowdrop) (University of Krakow, Poland)
- **Lily** (PRI, the netherlands)
- **Rose** (University of Krakow, Poland)
- **Yacon** (University of Prague, Czech republic)
- **Coconut** (RDA, South Korea)
- **Spruce** (Slovac acedemy of science)
- **Pine** (Slovac acedemy of science)
- **Ash** (CNR, Firenze, Italy)
- **Byrsonima** (UFLA, Brasil)
- **Callerya** (INTAS , China)
- **Stevia** (KU Leuven)
- **Raspberry** (FTRI, Italy)
- **Dragonfruit** (KU Leuven)

Provide facilities and training: Global Network of Cryo-Collections

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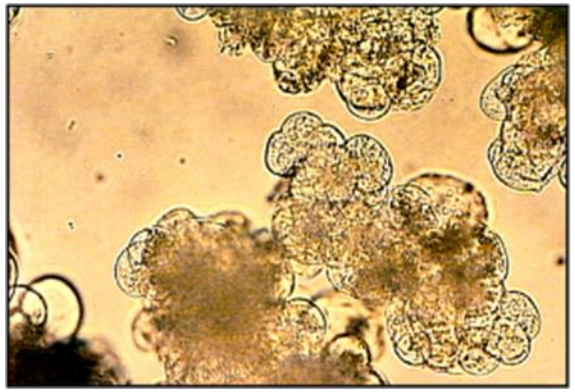
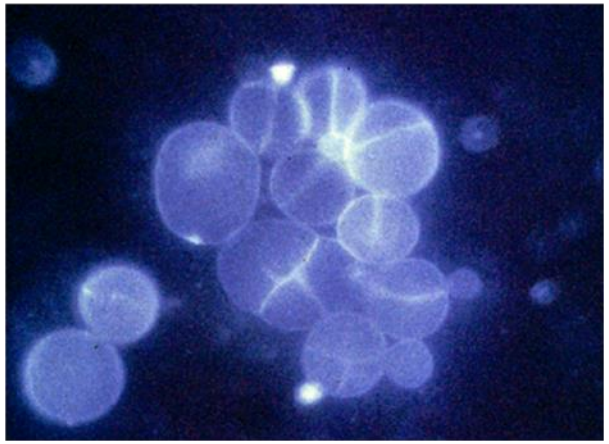
Other applications of cryopreservation

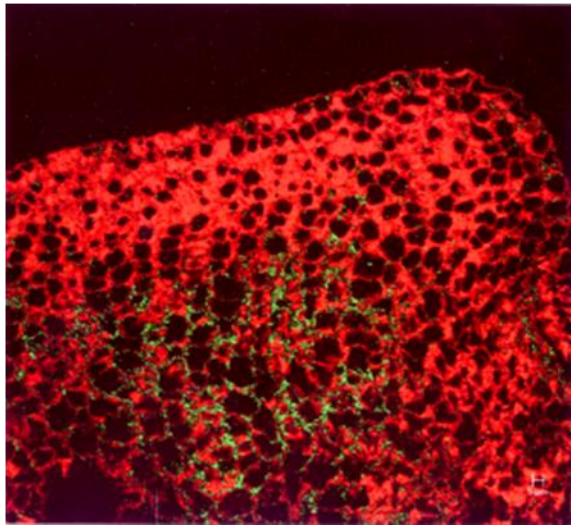
Long term storage of specific cell lines





Other applications of cryopreservation

Eradication of virusses



Other applications of cryopreservation

Breeding tool

Other applications of cryopreservation

Storage of clean stock cultures

Conclusions

- The conservation and sustainable utilization of plant genetic resources are the keys to improving agricultural productivity and sustainability
- Different storage methodologies are available; choice depend on species, available plant materials and facilities
- For long term conservation, cryopreservation should be considered for vegetative materials as well as for seeds
- Cryopreservation can also be used for eradication of viruses (and other microorganisms), as breeding tool, and as commercial stock deposit

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