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Alliance Bioversity & CIAT

CGIAR

Introduction to plant cryopreservation

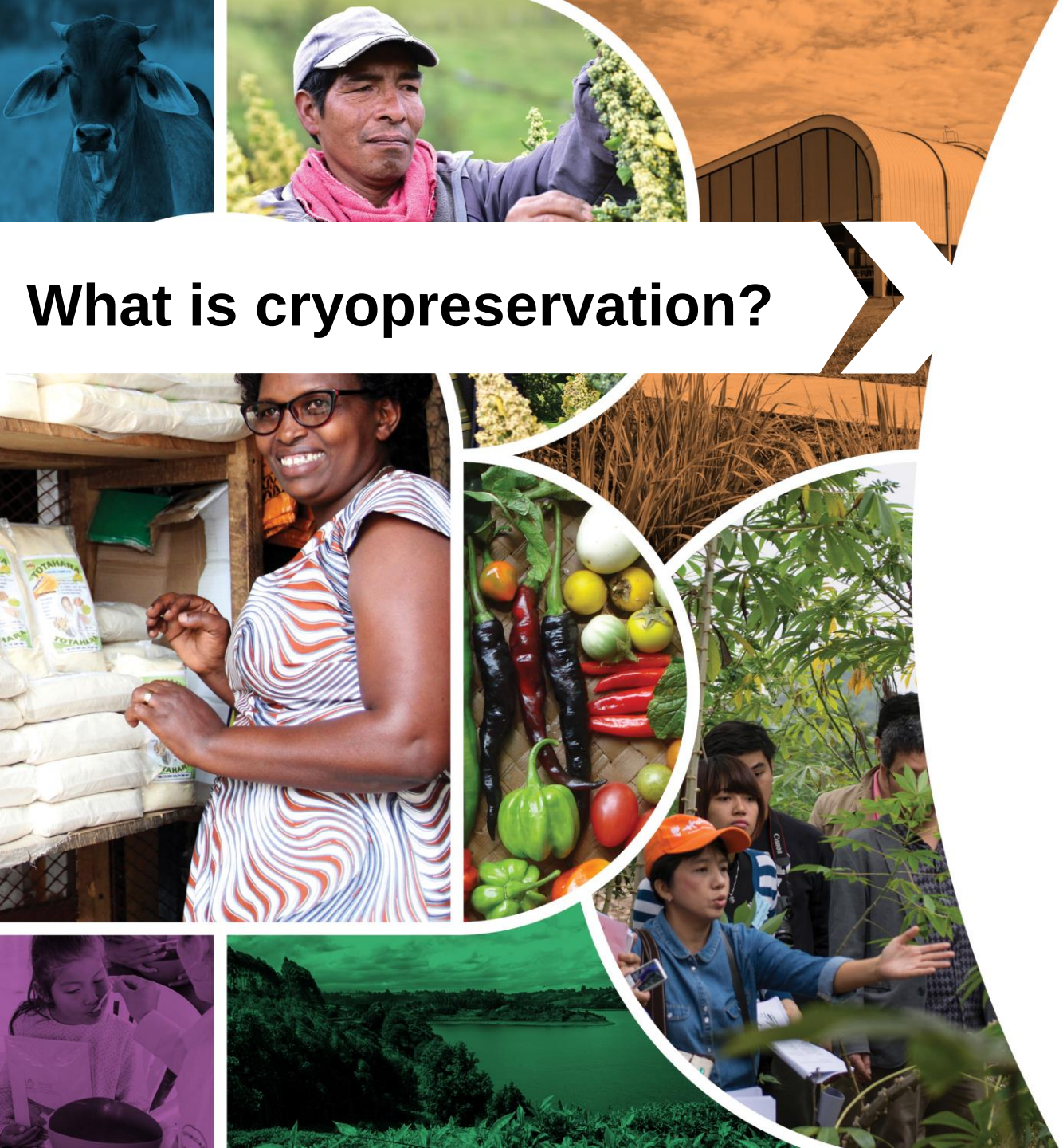
European Cooperative Programme for Plant Genetic Resources

ECP//GR

Bart Panis

Principal researcher Alliance of Bioversity International and CIAT/ KU Leuven

What is cryopreservation?



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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)

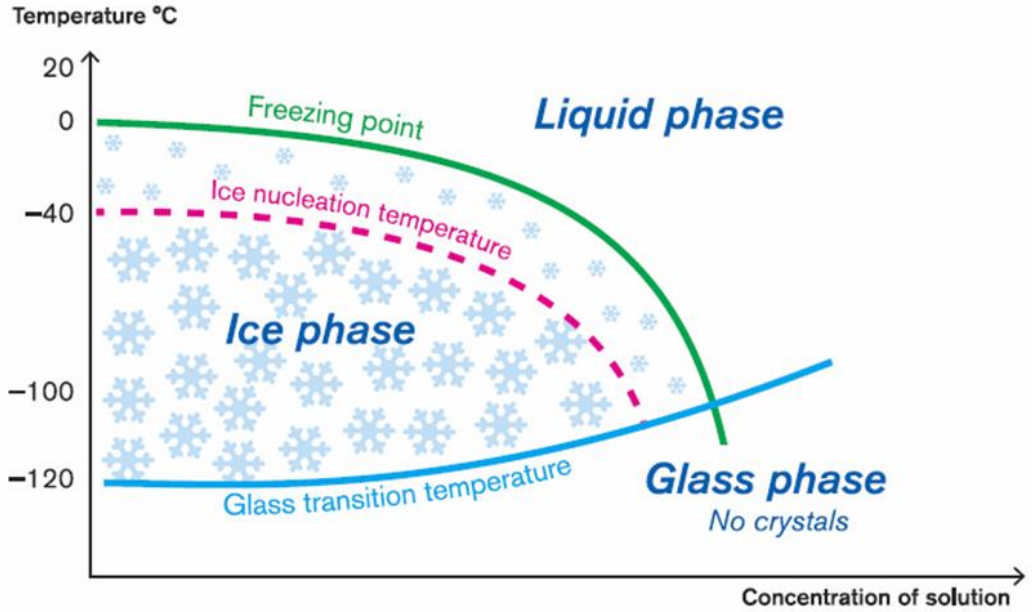
3/ **Dehydration related effects** (In nature, during cryopreservation when slow freezing rates are applied). Results in solution and mechanical effects

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

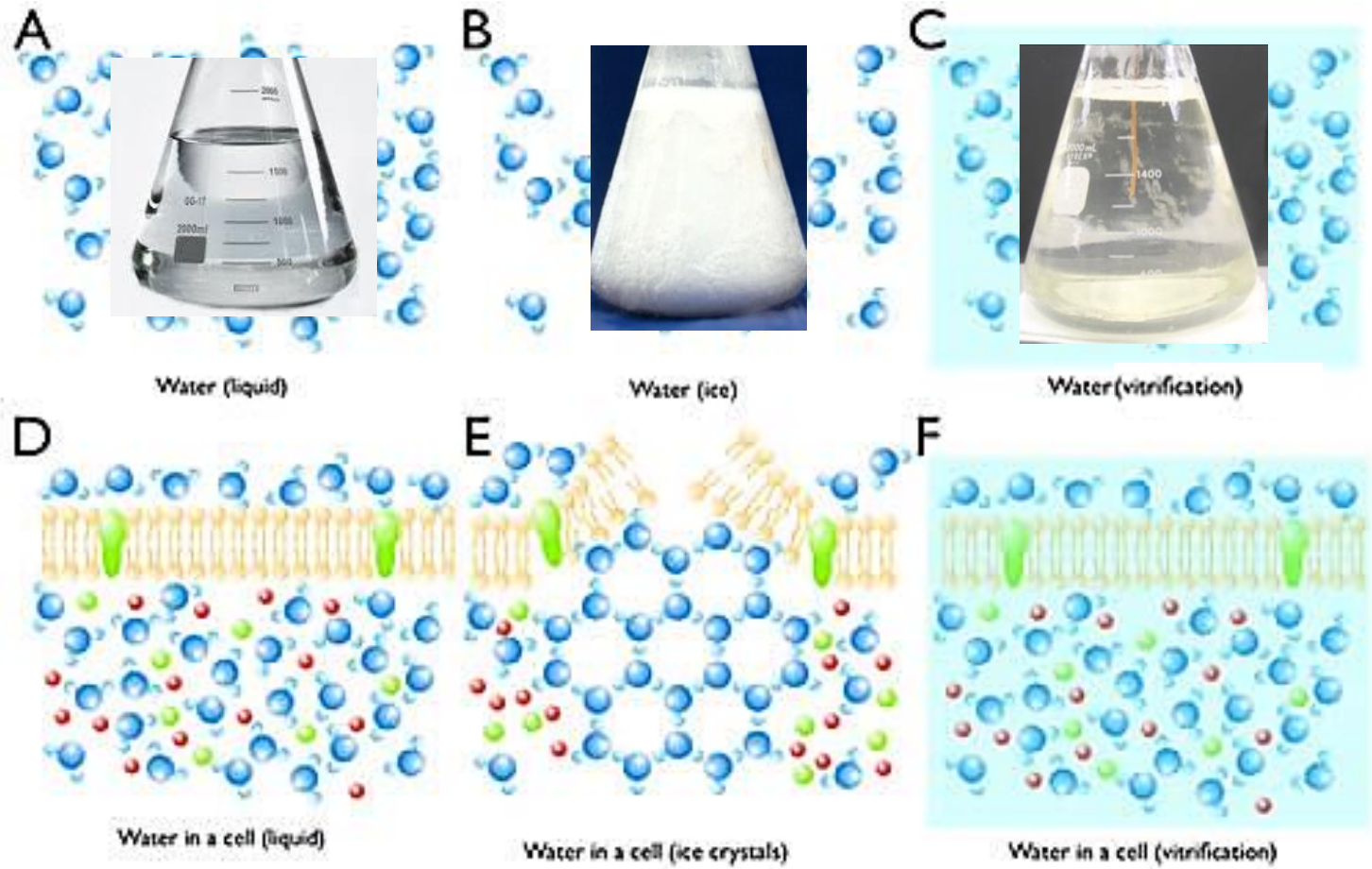
⇒ Mechanical disruption of protoplasmatic structure, loss of semi-permeability

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

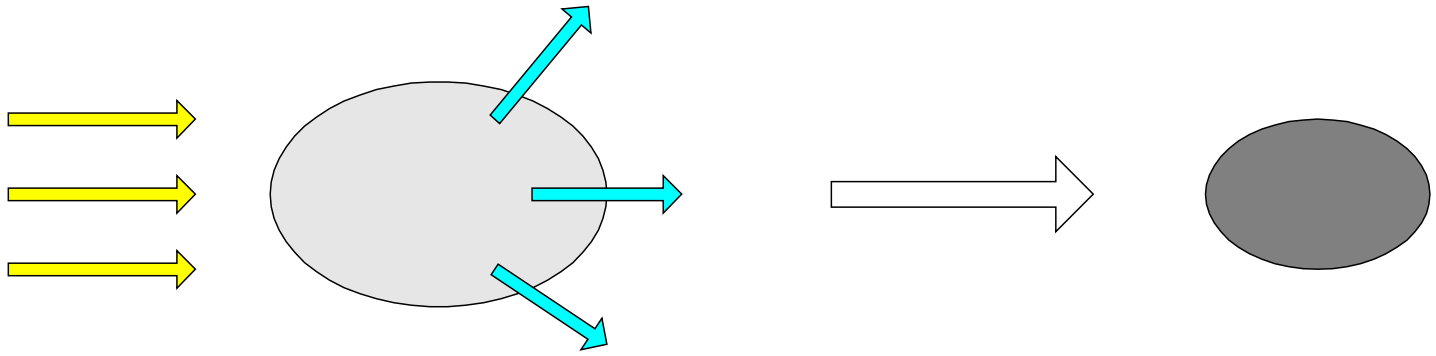


HOW???

1/ Concentration of cellular solution

2/ Rapid cooling and thawing rates

1. Concentration of cellular solution through air drying

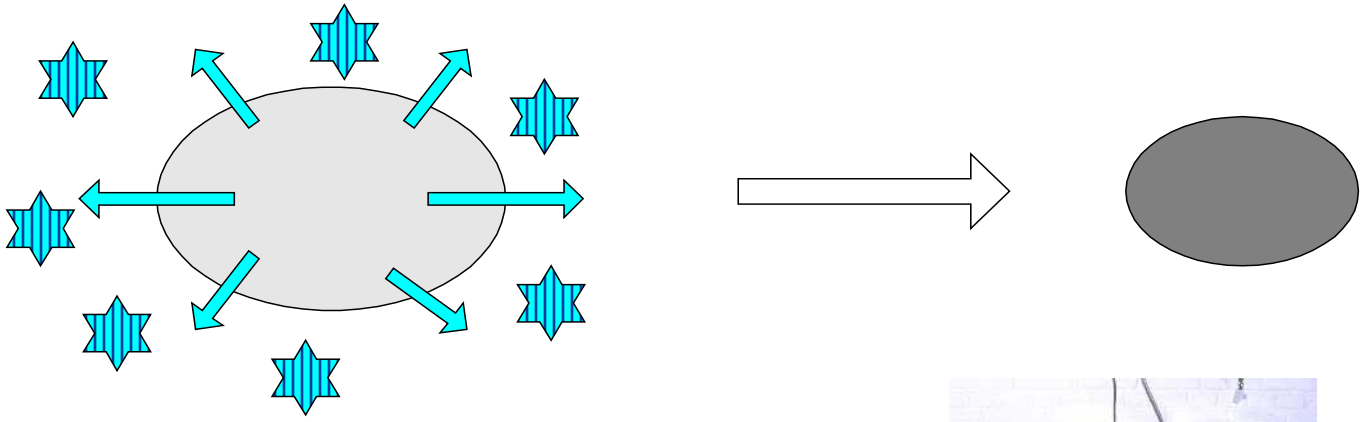


- Sterile air from laminar air flow cabinet

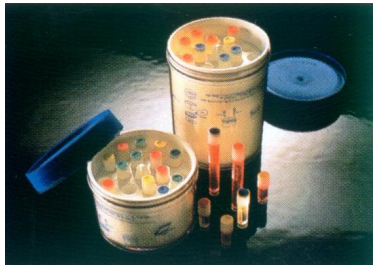
- Dry silica gel in a closed container

2. Concentration of cellular solution through freeze dehydration

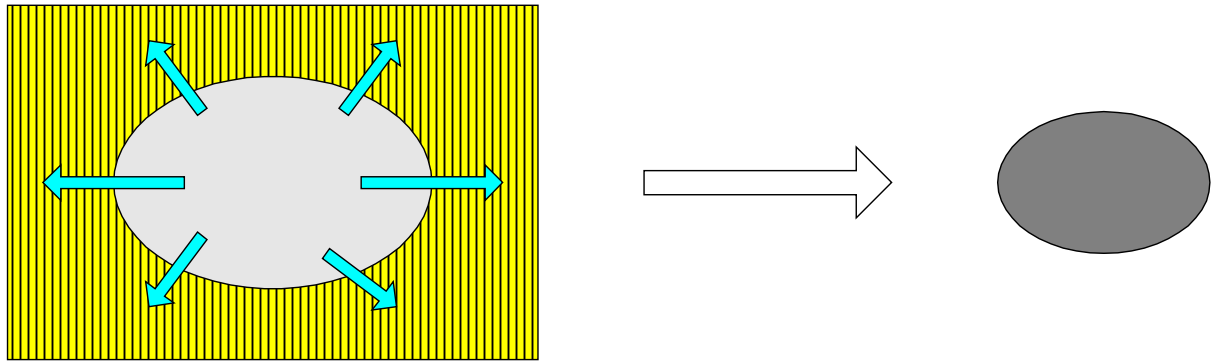
Cooling rates : 0.3 to 10°C/min until -30 to - 50°C



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



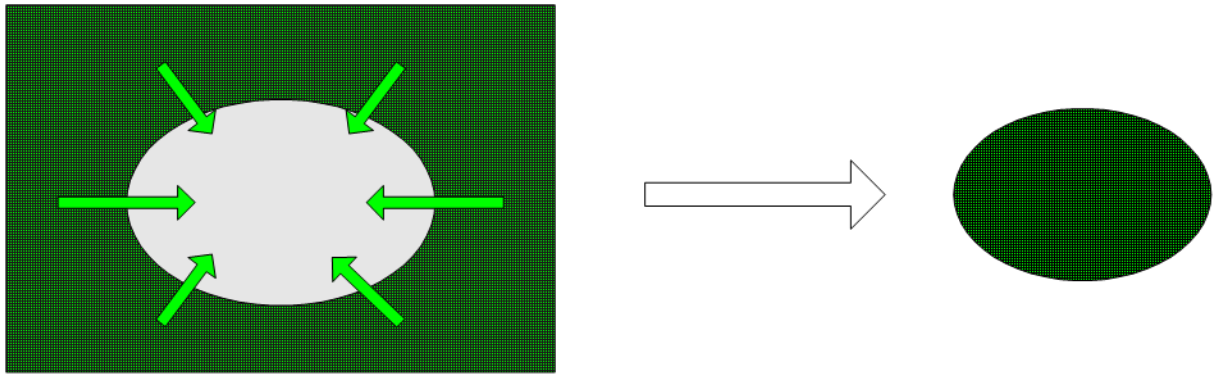
3. Concentration of cellular solution through osmotic dehydration



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



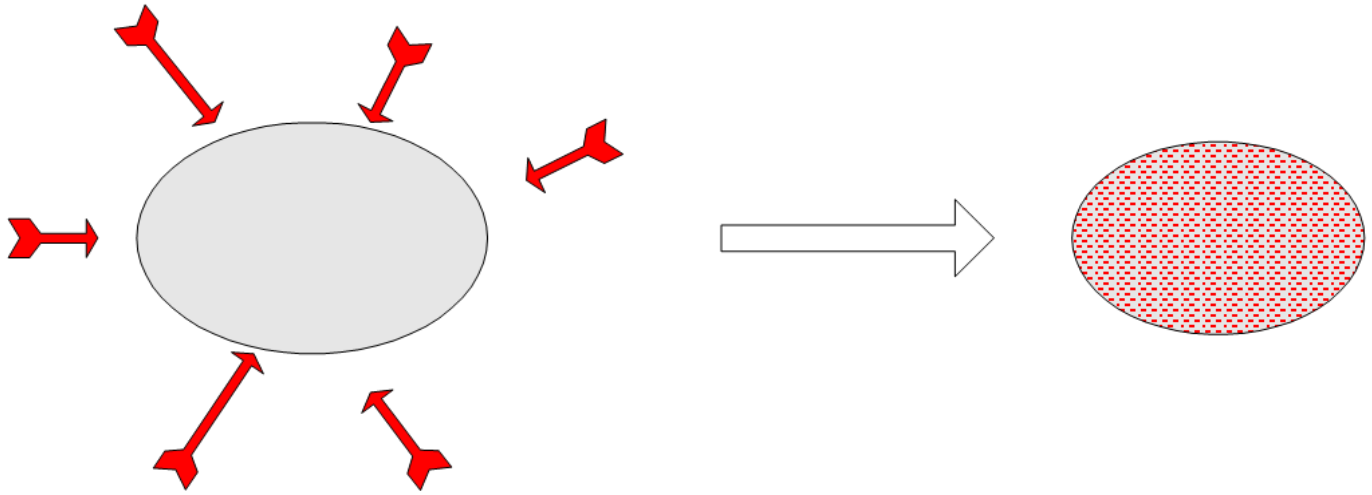
Colligative effect of penetrating cryoprotective substances

DMSO

Glycerol amino acids

EG at high temperature (RT)

5. Concentration of cellular solution through adaptive metabolism

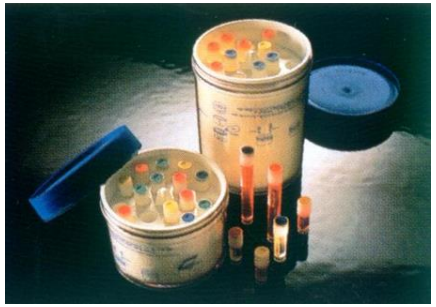


Induced by temperature changes, changes in light regime, osmotic changes, ABA,…..

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

Induction is genetically defined

Different cryopreservation protocols



?

Methods for cryopreservation

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

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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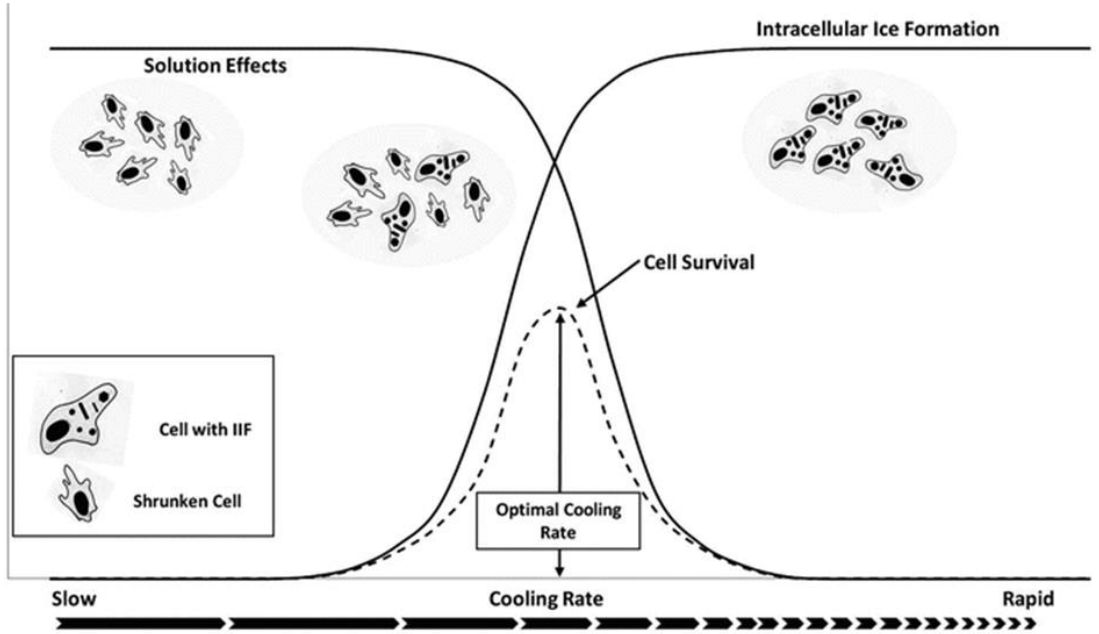
Classical (slow) freezing protocol

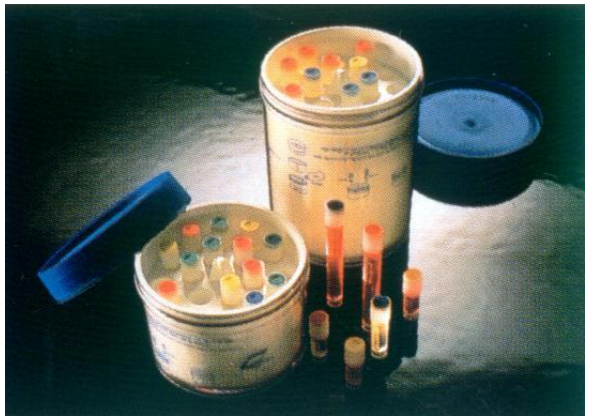
- Cold hardening and/ or Osmotic dehydration and/or Sugar hardening
- Penetrating cryoprotectants (often DMSO)+ non-penetrating cryoprotectants
- Freeze dehydration at 1°C /min to -35°C

Parameters to be optimised

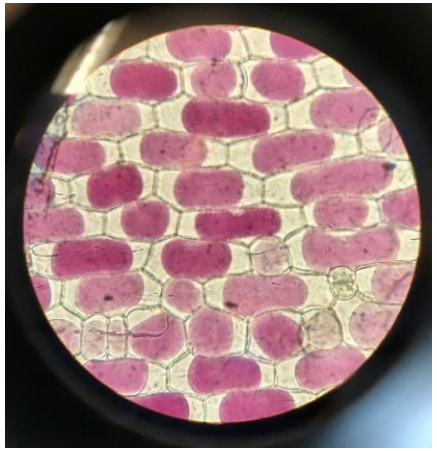
- Hardening
- Cryoprotective mixture (Often including DMSO)
- Cooling rate
- Holding temperature

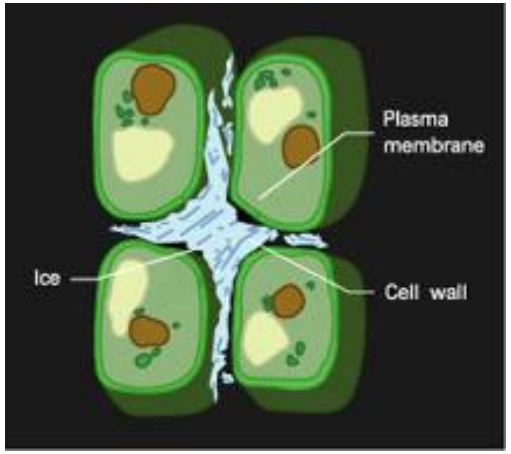

Intracellular Ice Formation
Solution Effects
Cell Survival
Cell with IIF
Shrunken Cell
Optimal Cooling Rate
Slow
Cooling Rate
Rapid


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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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,…)

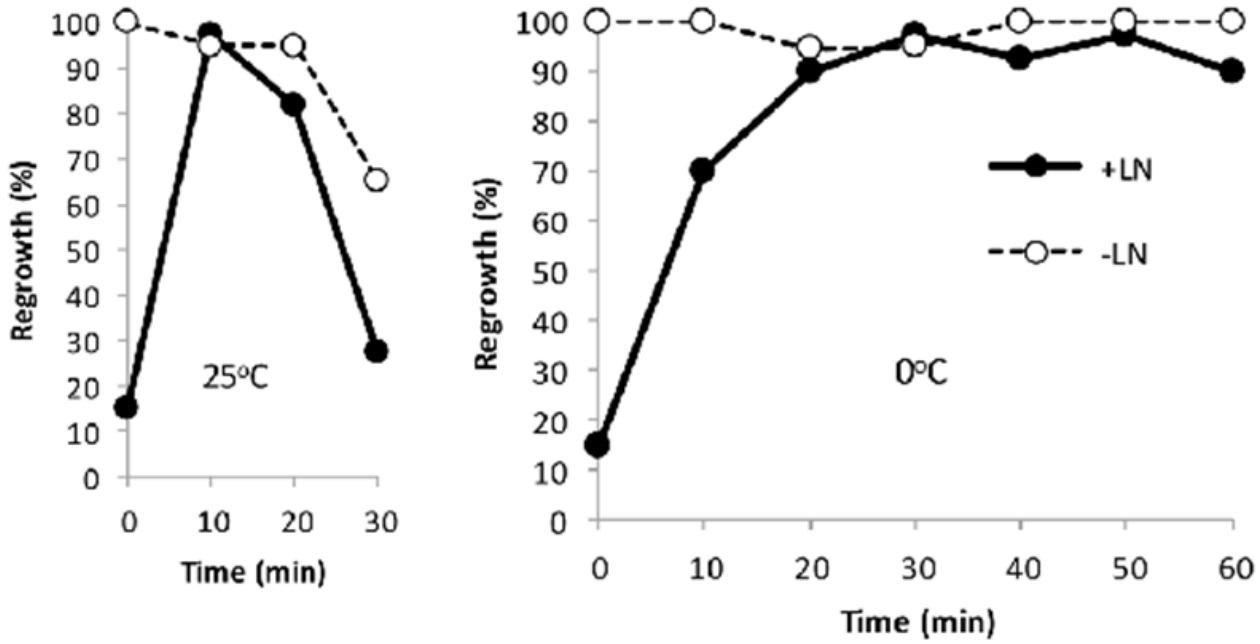

Fig.1. Effect of exposure time to PVS2 at 25 or 0 °C on recovery growth from wasabi shoot tips cooled to -196 °C by vitrification. Shoot tips (1 mm size) were precultured with 0.3 M sucrose for 1 d and then treated with a mixture of 2 M glycerol plus 0.4 M sucrose (LS solution) for 20 min at 25 °C. These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

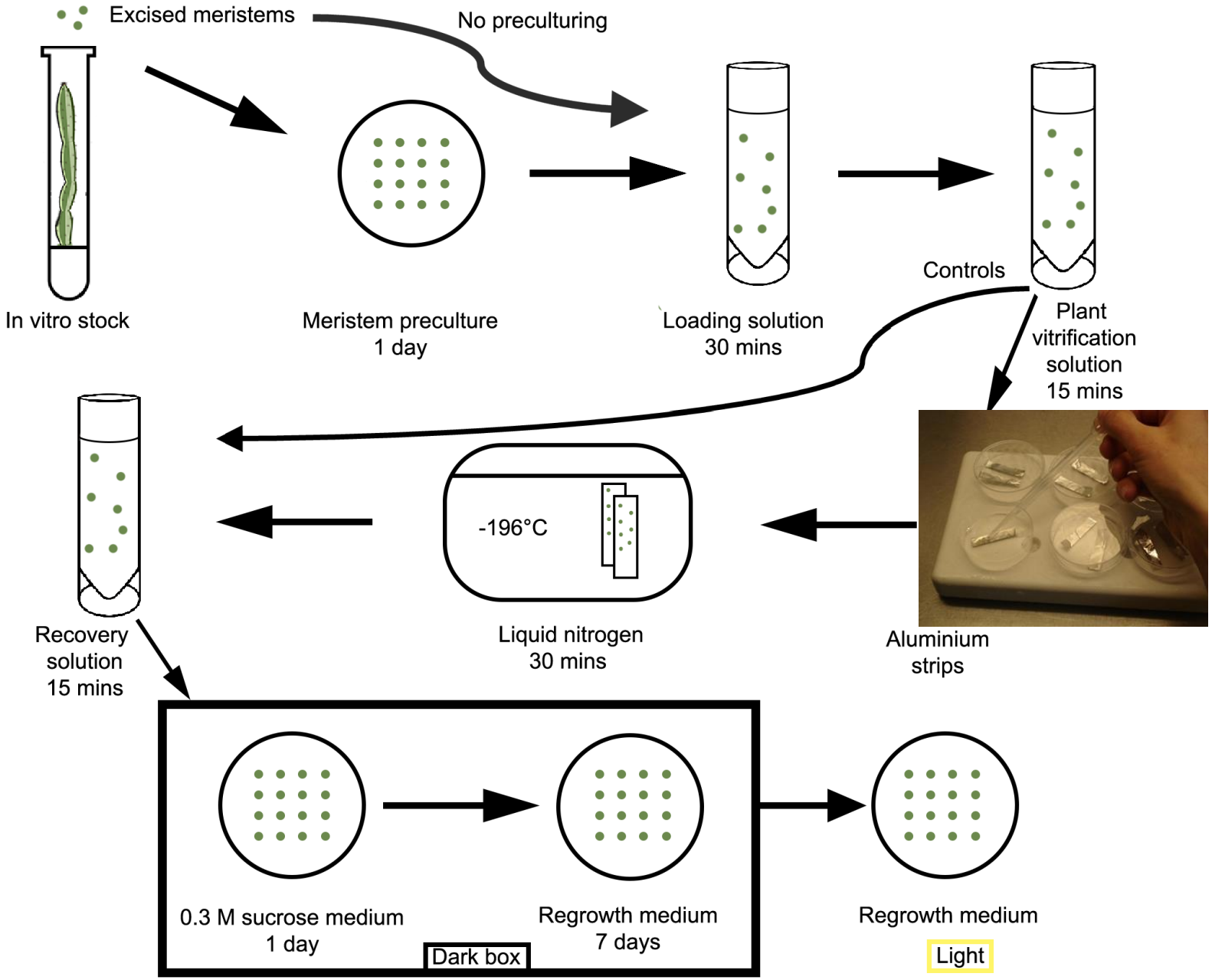
- Cryotubes (about 6°C/sec)
- Semen straws (about 60°C/sec)(potato)
- Droplet vitrification (about 130°C/sec)

Droplet vitrification

Survival

≠

Regeneration



Cryopreservation for storage of genetic resources ?

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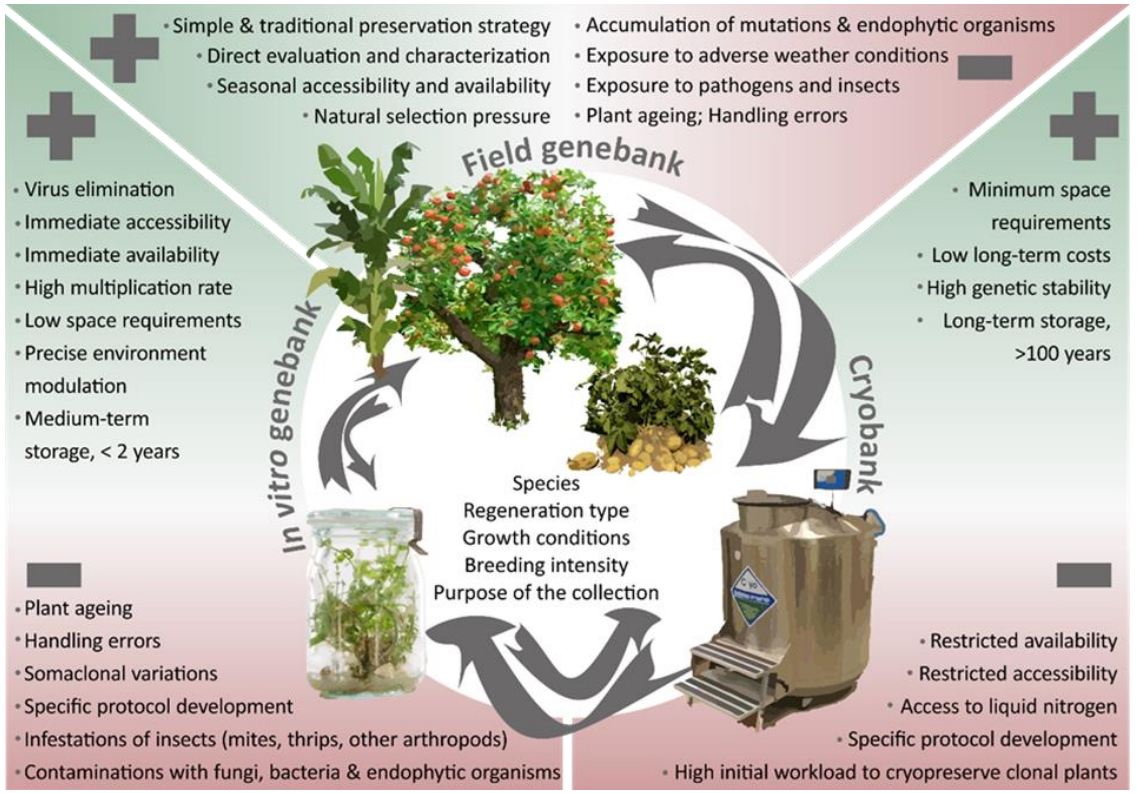

Methods of conservation

- *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

Methods of conservation

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  - 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)

IPK potato collection, Germany

Bioversity International in vitro banana collection, Belgium

Bioversity International Cryobank, Belgium

Cryopreserved collections

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FEASIBILITY STUDY FOR A SAFETY BACK-UP CRYOPRESERVATION FACILITY

INDEPENDENT EXPERT REPORT: JULY 2017

Australian Government
Australian Centre for International Agricultural Research

Federal Ministry for Economic Cooperation and Development

Schweizerische Eidgenossenschaft
Confédération suisse
Confederazione Svizzera
Confederaziun svizra

Swiss Agency for Development and Cooperation SDC

Bioversity International

CGIAR

CIP International Potato Center

CROP TRUST

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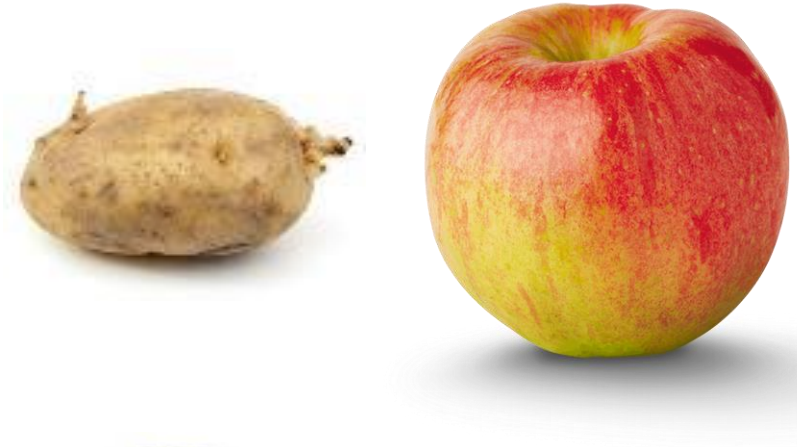
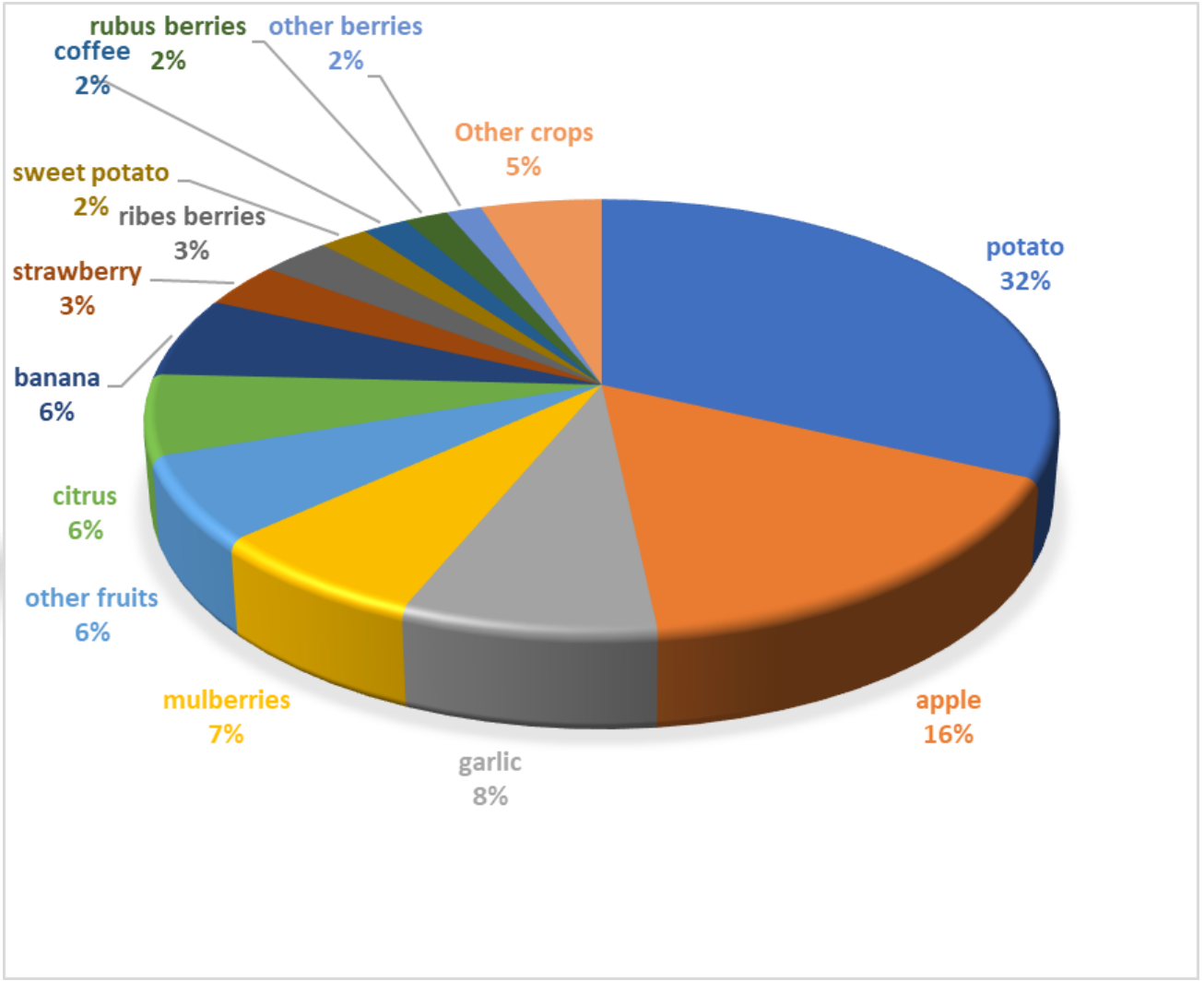
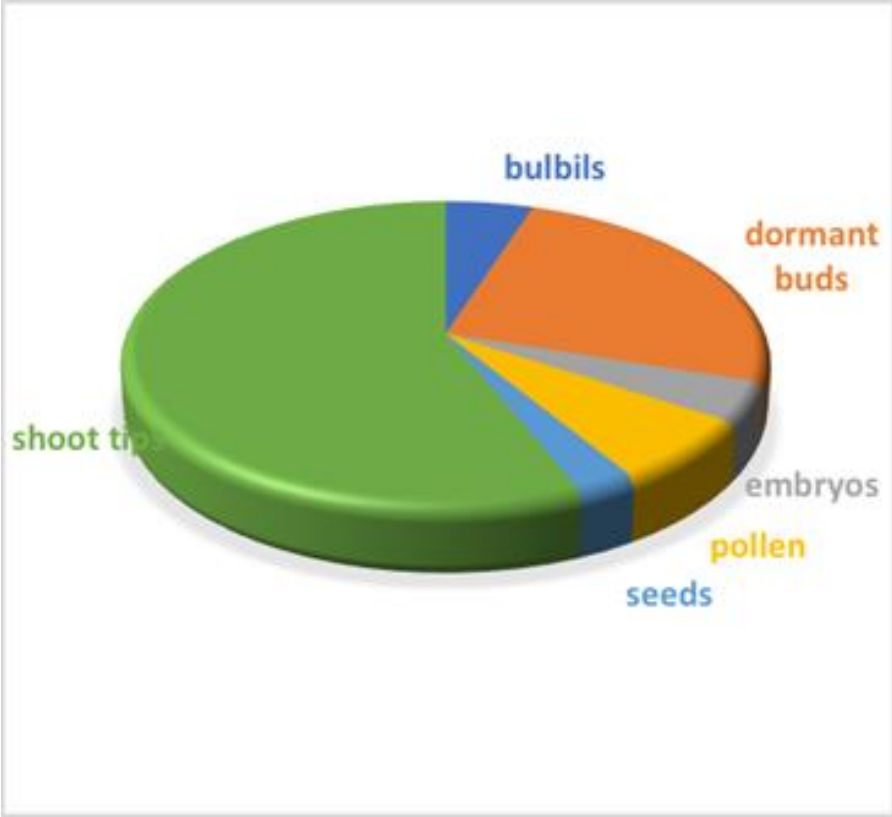
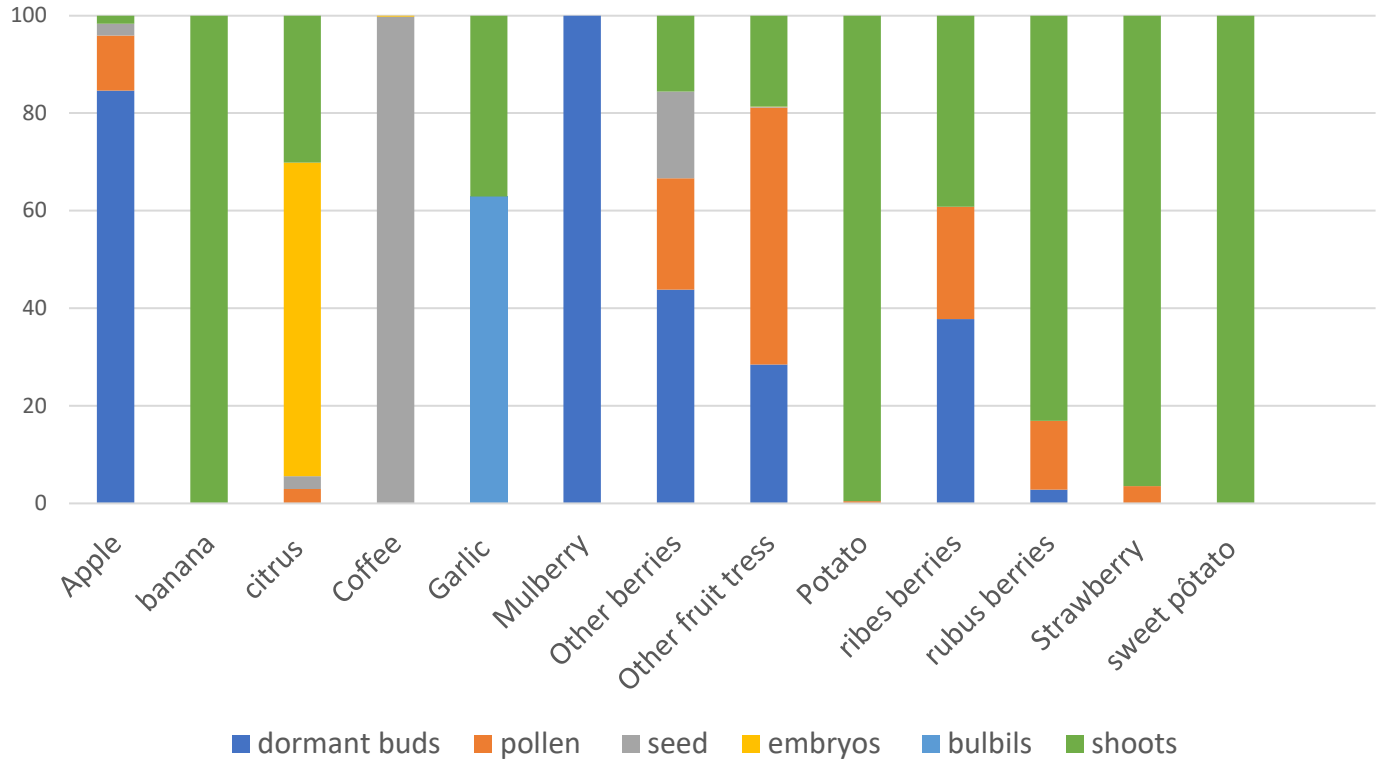
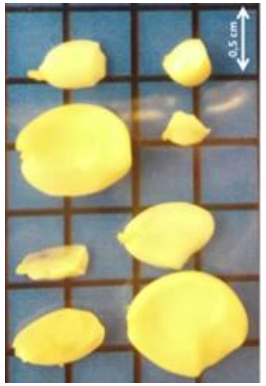
Only 17 crops have cryopreserved collections of more than 100 accessions !






bulbils
dormant buds
shoot tip
embryos
pollen
seeds

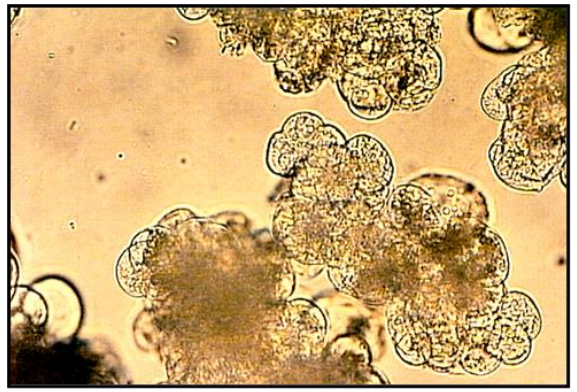

100
80
60
40
20
0
Apple
banana
citrus
Coffee
Garlic
Mulberry
Other berries
Other fruit tress
Potato
ribes berries
rubus berries
Strawberry
sweet pôtato
dormant buds
pollen
seed
embryos
bulbils
shoots

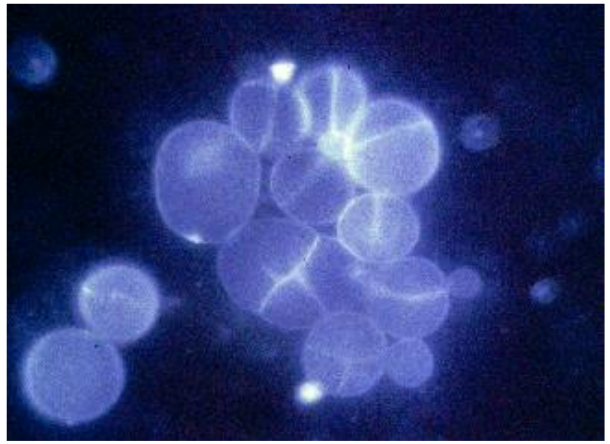

0.5 cm

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

Other applications of cryo

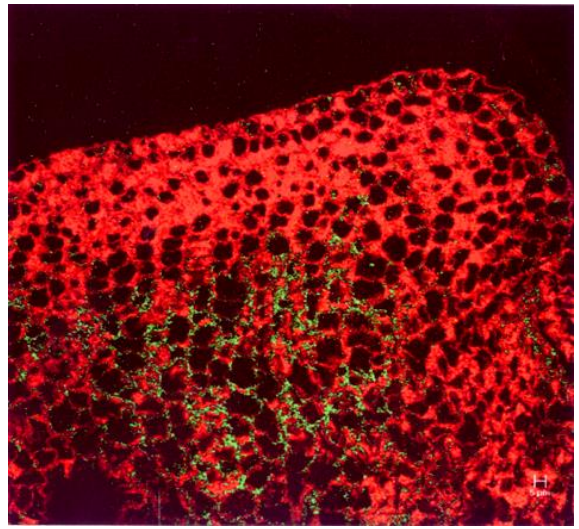
Long term storage of specific cell lines





Other applications of cryo

Eradication of viruses



Other applications of cryo

Breeding tool

Other applications of cryo

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

Acknowledments Partnerships

- Natalia Sleziak
- Hans Krohn
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- Bart Piette
- Hannes Wilms
- Ines van den houwe
- Elena Popava

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Australian Centre for International Agricultural Research

Federal Ministry for Economic Cooperation and Development

Schweizerische Eidgenossenschaft
Confédération suisse
Confederazione Svizzera
Confederaziun svizra
Swiss Agency for Development and Cooperation SDC

cost
European Cooperation in Science and Technology

Belgium
partner in development

IRD
Institut de recherche pour le développement

BILL & MELINDA GATES foundation

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Thanks!