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An introduction to plant cryopreservation

Bart Panis

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,……)

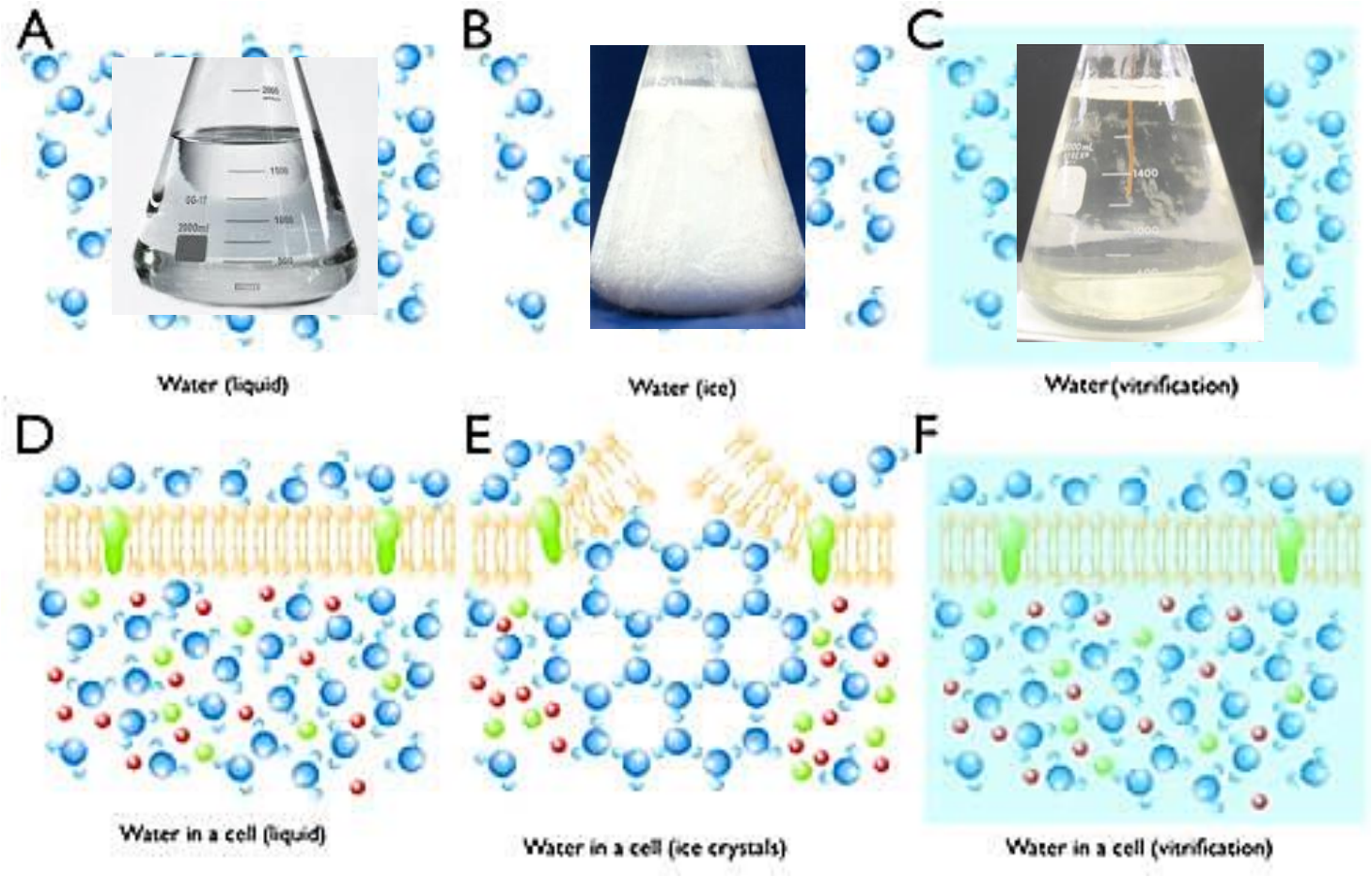
2/ Mechanical effects of extracellular ice crystals at cell surfaces (breaking of tissues, disconnection of cells)

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

Vitrification

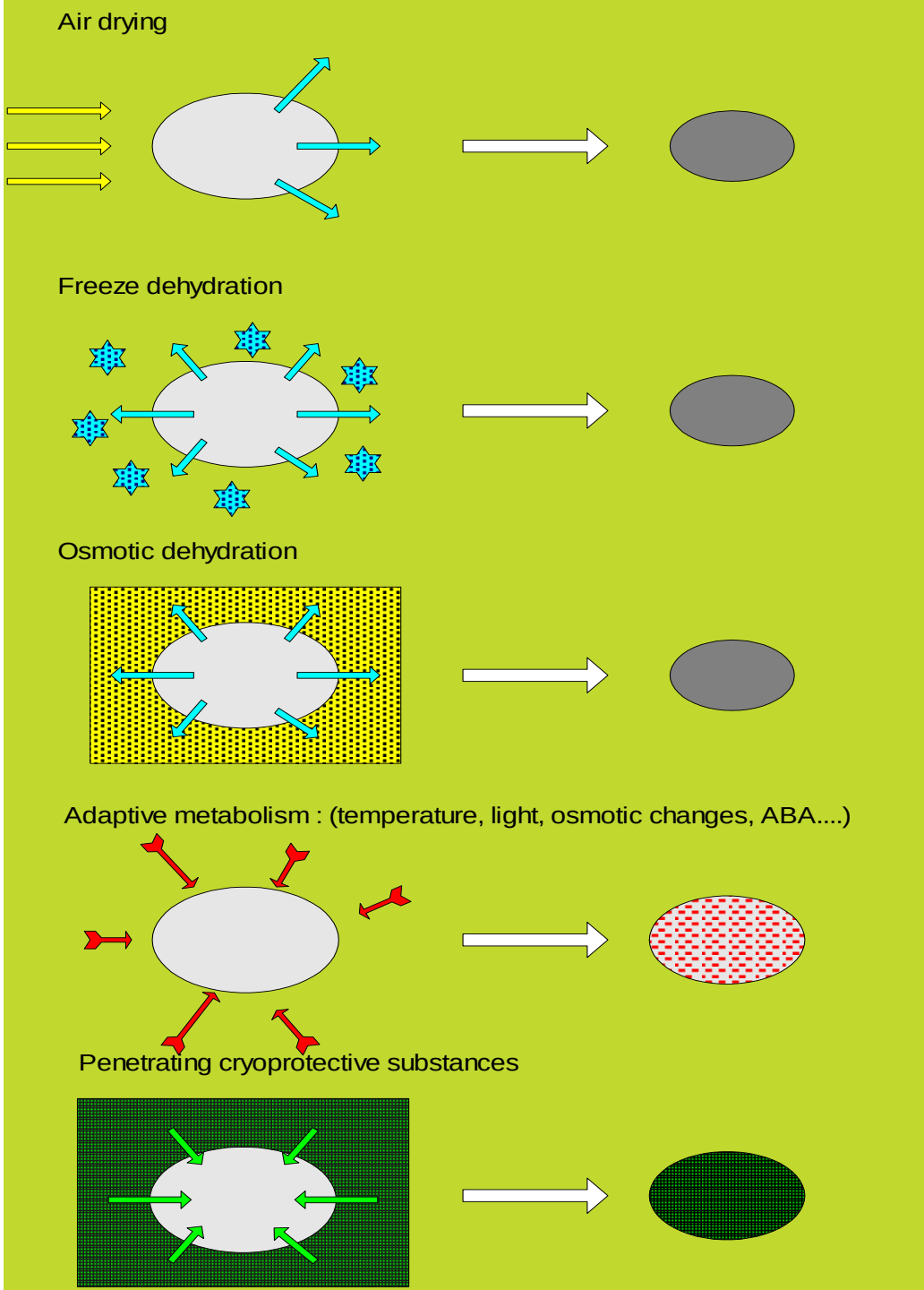


Prevention of intracellular ice crystal formation. through ‘vitrification’

HOW???

1/ Concentration of cellular solution

2/ Rapid cooling and thawing rates



- **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)

Different cryopreservation protocols

Methods for cryopreservation

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

Cryopreservation of dormant buds

Cecil Stushnoff, 1987 (dormant bud cryopreservation of apple)

Cold Hardening (Dormant field material)

Air dehydration at -5°C to 25-35% MC

Freeze dehydration at -1°C/hr to -35°C – hold 24hr

<u>Advantage no in vitro phase: grafting</u>

Parameters to be optimised

- Hardening
- Cooling rate
- Holding temperature

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,…)

Droplet-vitrification

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

COMBINATION OF

- Classical vitrification (with PVS2 or PVS3 or….)

AND

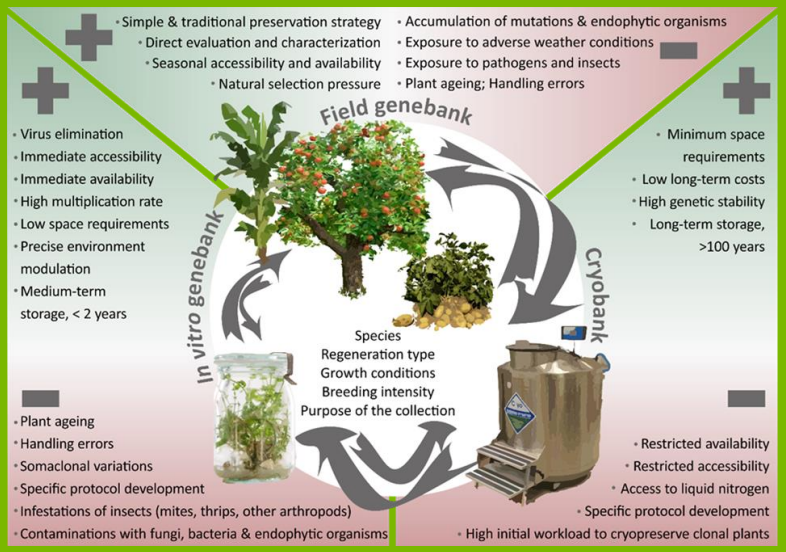
- The application of ultra fast freezing and ultra fast warming (to avoid respectively crystallization and cold crystallization).

How to obtain more rapid freezing rates ?

- Freezing in partially solidified nitrogen (sludge) which has a temperature of about –208°C (instead of –196°C in case of liquid nitrogen)

- 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)

Application 1. 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

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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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Methods of conservation

- *In situ* : Conservation in ‘normal’ habitat
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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)

State of the art of Plant cryopreservation

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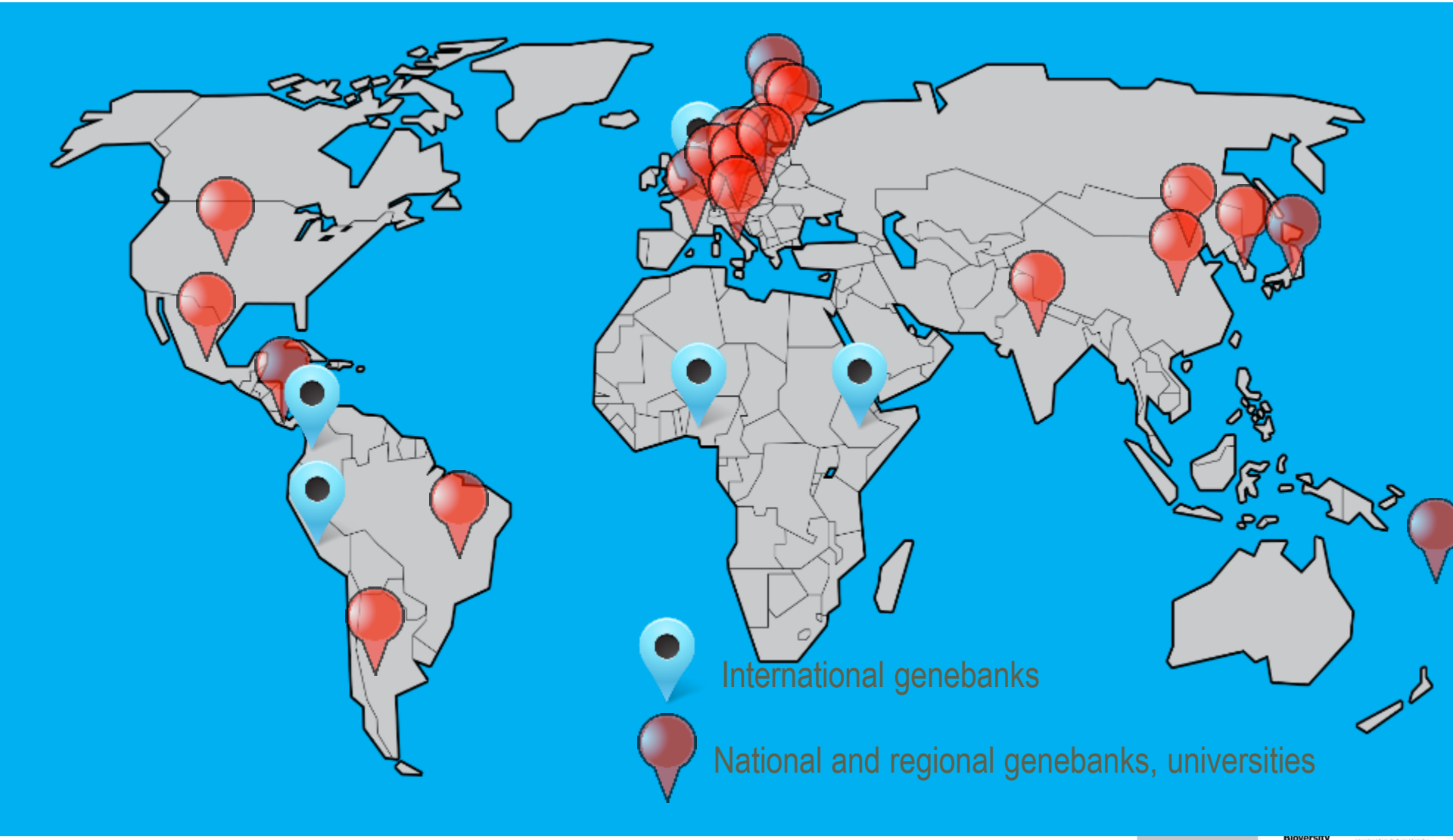
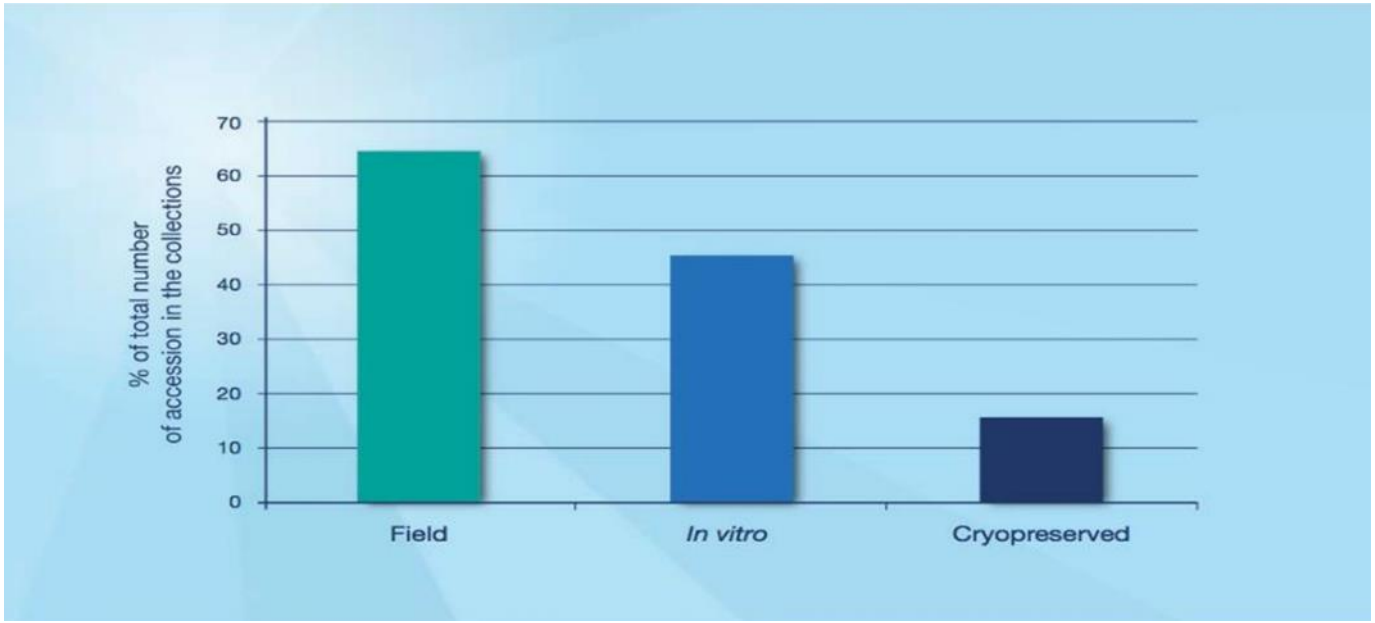
Survey was sent to 26 organizations around the world holding existing or emerging cryo-collections

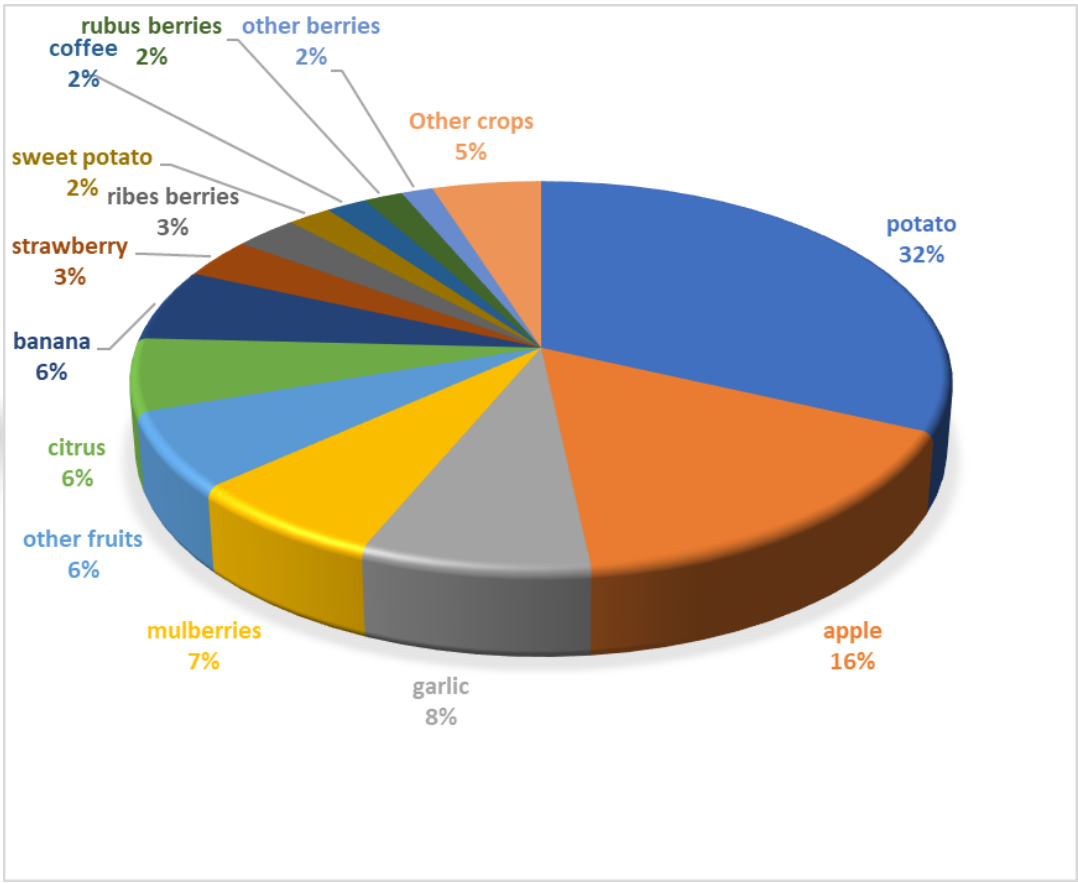


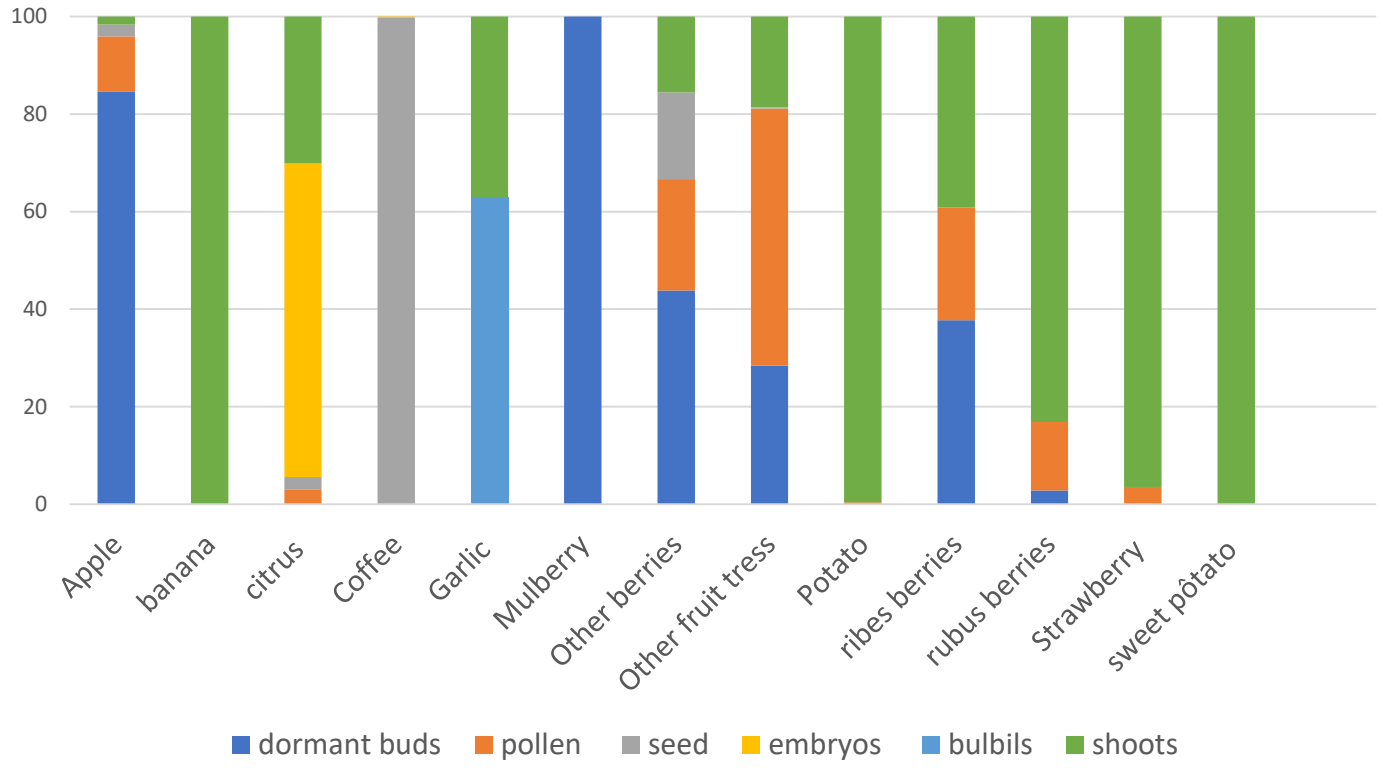
Status of cryopreserved crop collections

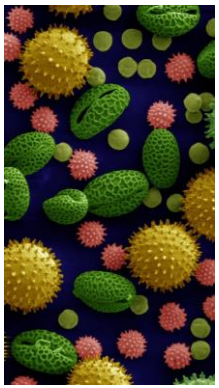
- 15 institutes together hold 9,650 accessions of 30 crops in cryopreservation
- This constitutes only 16% of the total number of accessions they collectively hold of these crops.
- The majority of the accessions are maintained in the field (66%) and/or *in vitro* culture (46%).

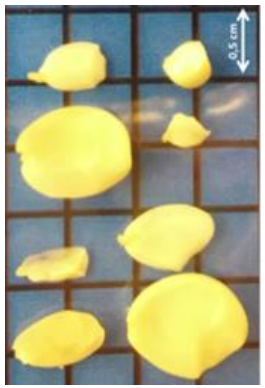


Only 17 crops have cryopreserved collections of more than 100 accessions !









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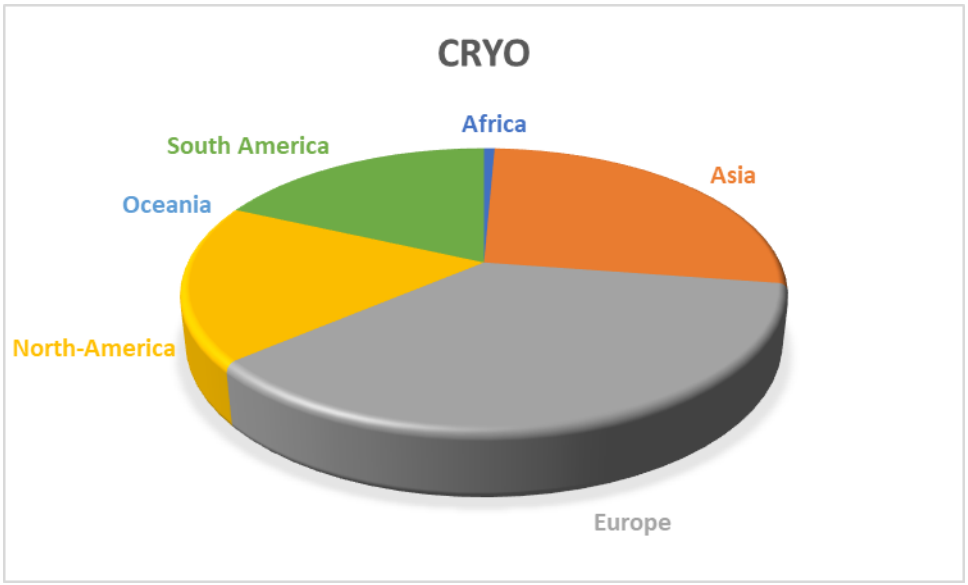
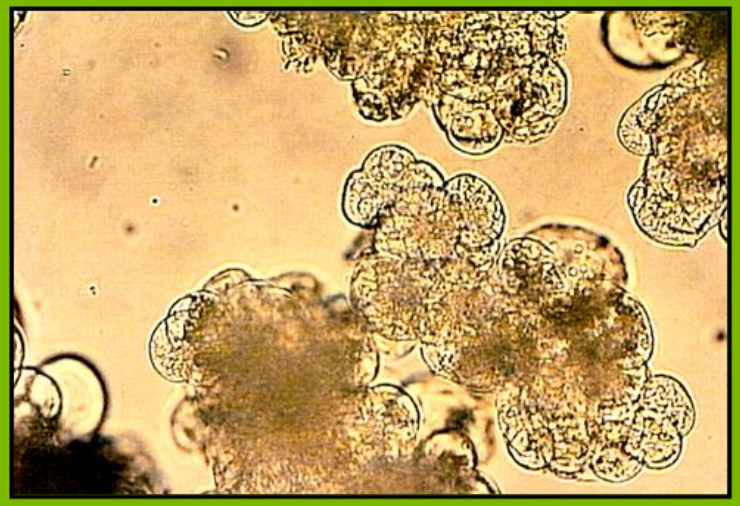
Institute	N° of Acc.	Crop	Cryopreservation Method
Bioversity International, Leuven, Belgium	1100	Banana	• Droplet vitrification
Association FOrêt-CELlulose (AFOCEL), France	440	Elm	• Dormant bud freezing
International Center for Tropical Agriculture (CIAT), Cali, Colombia	480	cassava	• Droplet vitrification • 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

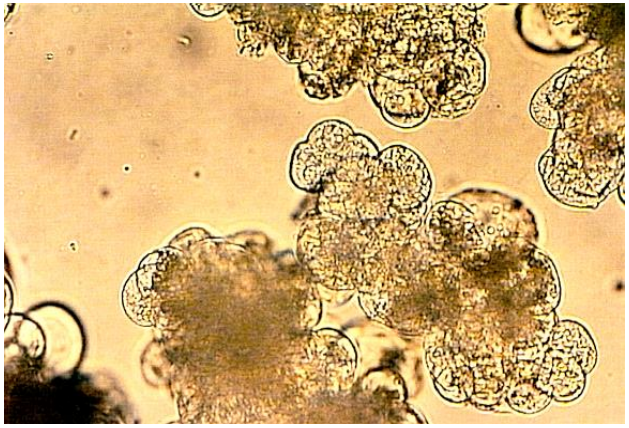

CRYO
Africa
South America
Asia
Oceania
North-America
Europe

Application 2. Long term storage of specific cell lines



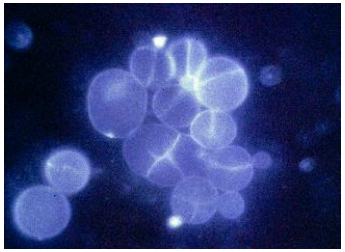
Example: cryopreservation of banana embryogenic cells

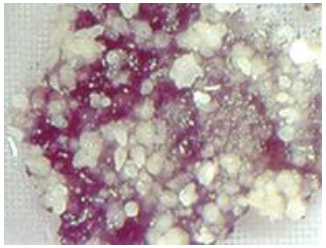
Problems related to the use of embryogenic cell suspensions in banana

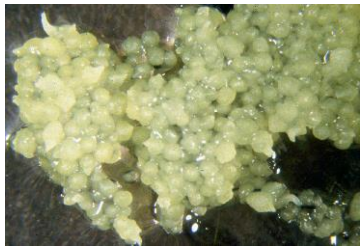


- Their initiation is difficult and time consuming (up to 2 years !)
- Once initiated, they can be subject to :
  - somaclonal variation
  - microbial contamination
- Prolonged culture periods result in loss of morphogenic capacity

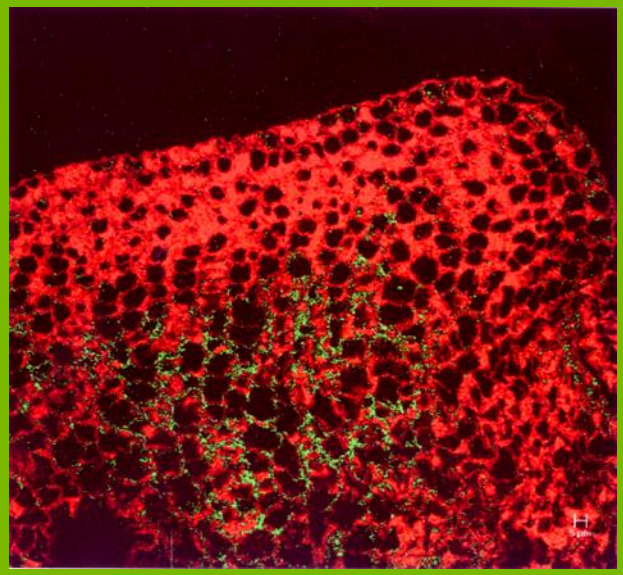
Regenerable suspensions should be safely stored in liquid nitrogen, since they are the material of choice for :







Application 3. Eradication of virusses



Examples of cryotherapy for pathogen eradication (Wang et al., 2009)

Table 2 Comparison of cryotherapy of shoot tips (Cryo) and meristem tip culture (Meri) for their efficiency in pathogen eradication

		Plant regeneration from shoot tips (%)		Pathogen-free regenerants (%)		
Plant	Pathogen	Meristem	Cryo	Meristem	Cryo	References
Banana	CMV	100	76	4	34	Helliot *et al.* (2002)
Banana	BSV	100	76	76	90	Helliot *et al.* (2002)
Grapevine	GVA	75	60	12	96	Wang *et al.* (2003)
Potato	PLRV	55	87	56	85	Wang *et al.* (2006)
Potato	PVY	55	87	62	93	Wang *et al.* (2006)
Prunus hybrid	PPV	85	50	19	50	Brison *et al.* (1997)
Raspberry[a]	RBDV	60	30	0	35	Wang *et al.* (2008)
Sweet orange[b]	HLB	69	85	25	98	Ding *et al.* (2008)
Sweet potato[c]	SPCSV	100	87	100	100	Wang & Valkonen (2008b)
Sweet potato[c]	SPFMV	100	87	10	100	Wang & Valkonen (2008b)
Sweet potato[c]	SPCSV + SPFMV	100	87	7	100	Wang & Valkonen (2008b)
Sweet potato[d]	SPLL	100	85	10	100	Wang & Valkonen (2008a)

BSV, banana streak virus; CMV, cucumber mosaic virus; GVA, grapevine virus A; HLB, huanglongbing bacterium; PLRV, potato leaf roll virus; PVY, potato virus Y; RBDV, raspberry bushy dwarf virus; SPCSV, sweet potato chlorotic stunt virus; SPFMV, sweet potato feathery mottle virus; SPLL, sweet potato little leaf phytoplasma.

[a]Shoots were subjected to thermotherapy followed by cryotherapy of the excised shoot tips.

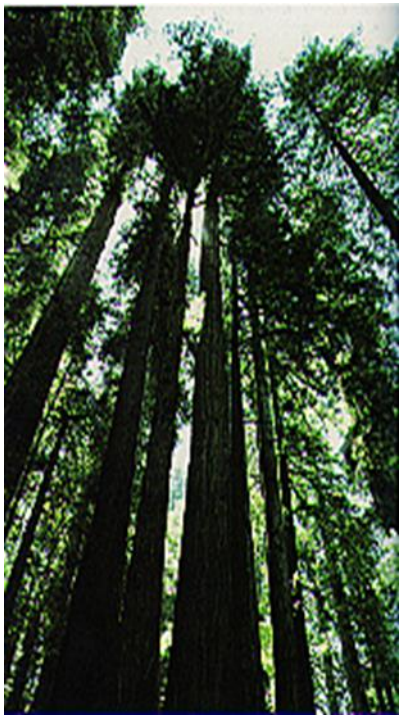
[b]Applied also to another accession of sweet orange, Beijing lemon, mandarin and pummelo, which resulted in HBL-free regenerants at 93%, 91%, 93% and 94% frequency, respectively.

[c]Shoot tips size 1.5 mm.

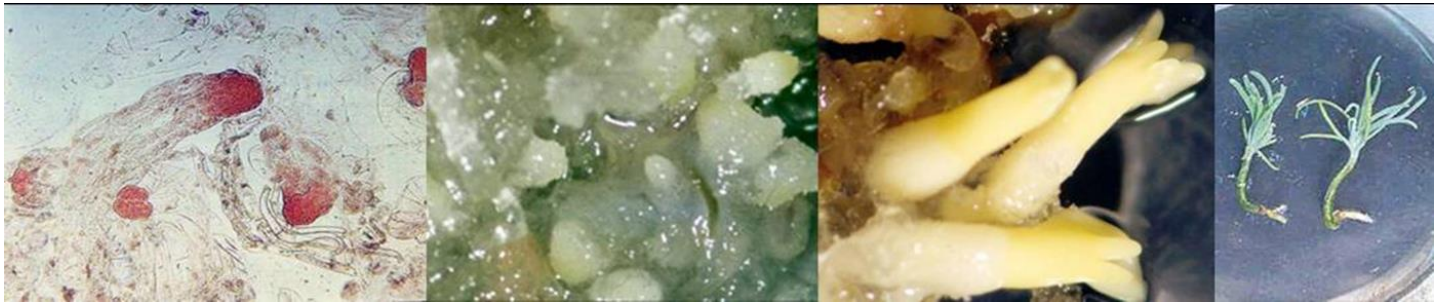
[d]Shoot tip size 1.0 mm.

Application 4. Breeding tool

Cryopreservation as a breeding tool



- Many adult trees (such as conifers) can not be vegetatively propagated (rejuvenation problem)
- Clonal propagation only possible through somatic embryogenesis (starting from seeds)
- It takes decades before the value of a selection/cross can be determined
- Storage of embryogenic cultures could meanwhile happen through cryopreservation



Application 5. Storage of clean stock cultures

Cryopreservation to store of clean stocks for the long term in a production environment

- Large amounts of independent in vitro cultures are being conserved by in vitro production companies

  – *Cultures in production.* It is adviced to store a “clean”, true to-type back-up *in case of problems of contamination, hyperhydricity and somaclonal variation

  – *Cultures that are “On hold”* . Putative interesting but not in production. Their maintenance is costly and and risks of loss.

A cryopreserved stock locally stored or in a specialized facility could provide a solution

Future of plant cryopreservation

Many efficient cryopreservation protocols for many species are available; are all problem solved?

- **Cryopreservation of some species remains difficult**
- Sometimes tissues survive cryopreservation but **do not grow out "normally"**
- Cryopreservation remains labor intensive and thus costly. Should be considered as a **long term investment**
- Presence of **endogenous microorganisms**

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Confederaziun svizra

Swiss Agency for Development
and Cooperation SDC

Belgium
partner in development

https://www.bioversityinternational.org/e-library/publications/detail/feasibility-study-for-a-safety-back-up-cryopreservation-facility-independent-expert-report-july-2017/

Thank you

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