In vitro genebanks and Cryogenebanks – Global status and Prospects
Conserving crops in situ and in genebanks

>80% of germplasm is conserved by seeds...

Clonally propagated crops are conserved in the fields or *in vitro*...
Background

There are crops in Annex 1 and Article 15 of PGRFA that cannot be conserved by seeds in conventional seed genebanks.

- Seedless crops
- Crops that do not breed true from seeds
- Crops with recalcitrant or short-lived seeds
Critical Food and Nutrition Security Crops that Cannot be Conserved in Perpetuity by Seeds

Annually 1 billion tonnes of these crops are produced globally, valued at US$100 billion (FAOSTAT)
The Case for a Global Cryo-Collection

National Genebanks conserve about 6.6 million of the total 7.4 million accessions held worldwide.

11 CGIAR Genebanks collectively conserve >750,000 accessions.

The Svalbard Global Seed Vault contains >5000 crop species, farmers’ land races, breeding material and wild plants.
The Feasibility Study (2017)

Multi-partner and multi-disciplinary task force:

1: Assessment of current and future potential use

2: Analysis of the state of conservation of propagated and recalcitrant seed species

3: Policy and technical requirements for the location and operation

4: Costs of establishing and running a safety back-up cryopreservation facility
Focus of the study:
Plant Genetic Resources for Food and Agriculture

- Annex 1 crops;
- Article 15 crops;
- PGRFA in the existing cryobank collections

- Survey was sent to institutions that are holding cryopreserved collections or are ready to cryopreserve their PGRFA

- Number of accessions of vegetatively propagated crops and crops with recalcitrant seeds in major ex situ collections has been estimated using open information sources
Survey was sent to 26 organizations around the world holding existing or emerging cryo-collections.
Survey structure:

1. For crops that are routinely cryopreserved we have asked for
   - number of accessions currently held in the fields, *in vitro* and in cryobank
   - expected number of accession in the cryobank expected within 5 years

2. For crops with cryopreservation protocols under development we have asked for
   - number of accessions currently held in the fields and *in vitro*
   - do you expect cryopreservation protocol to be developed within 5 years?
   - how many accessions will be cryopreserved within 5 years?

3. Duplication of cryopreserved collections.
   - where, what part of the collection can be duplicated under black box agreement?

4. Potential use of a safety back-up cryopreservation facility
   - if an international cryostorage facility became available in 2018 and your institute agreed to use it, would you have accessions with sufficient sample replicates ready to make a deposit?

5. What are the constrains that limit the rate at which you can cryopreserve crops / more accessions from the collection?
   - Insufficient budget
   - Lack of skilled personnel
   - Lack of equipment
   - Protocol issues
   - Other technical issues *specify*
   - Other issues *specify*
Part 1. Status of cryopreserved crop collections

- The 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%).
The Feasibility Study Conclusions:
Narrow Range of Crops in Cryo-Collections

Percentage representation of different crops in the cryopreserved collections of the 15 institutes (total 9,650 accessions)

- Potato
- Cassava
- Mulberry
- Banana & Plantains
- Allium
- Coffee
- Strawberry
- Mint
- Apple
- Citrus
- Sweet potato
- Taro
- Other crops*

*includes artichoke, hop & grapes, and other fruit trees & berries

Have collections >100 accessions
Part 1. Status of cryopreserved crop collections

• For the majority of crop collections held by the 15 institutes, less than 40% is cryopreserved. The only exception is banana and plantain.

• The scale of cryopreservation varies significantly among institutions.
Part 1. Status of cryopreserved crop collections

Major difficulties and constrains
Part 1. Status of cryopreserved crop collections

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.)
Part II. Data on vegetatively propagated crops

Annex 1 and Article 15

Information was compiled using the open sources:
- Global Crop Strategies
- Genesys database
- FAO: “Wiews” database
- FAO: “State of the genetic resources for food and agriculture” book
- Institution web-pages
- Reports to Crop Trust, including on regeneration projects
- Published reviews

Issues:
- Differences in numbers of accession between different sources;
- No clear separation between field and in vitro collections;
- Incorrect and frequently old data (webpages are not regularly updated)
- Duplicates are not separated

Current solution: for each crop
1. Compare different sources;
2. Use the most recent and complete source of information
Part II. Data on vegetatively propagated crops

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Current solution: for each crop
1. Compare different sources;
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Results: How many accessions of clonal/recalcitrant seed crops are there?

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

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

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

Current status

- 9,650 accessions are currently cryopreserved
- This number will potentially increased to 15,526 accessions by 2022
- 8 institutes are already interested in using the safety back-up cryopreservation facility. They expect to be able to deposit 6,217 accessions in 2019 and 11,412 by 2022 (one 800L tank?)

Potential

Approximately 400,000 accessions are conserved only in the field or in vitro culture, mainly in national genebanks.

Even assuming that only one in four accessions is distinct, there could be as many as 100,000 distinct accessions not yet cryopreserved and secured for the long-term.

Given the constraints in budget, infrastructure and personnel that many genebanks face, especially NARIs in developing countries, it is unlikely that these 100,000 accessions can be secured in cryopreservation without substantive support and capacity building.
Recommendations

1. A major global initiative is launched to accelerate the development and implementation of crop cryopreservation.
   • The Expert Group recommends a collaborative effort among researchers and genebanks that is focused on the specific technical and practical issues.

2. A cryopreservation facility is set up to accommodate the estimated 5,000 to 10,000 accessions.
   • These accession are arising from current, on-going cryopreservation activities at CGIAR and other genebanks identified in this study, that need safety backup over the coming five years.
   • Although the physical capacity requirements are modest, the Expert Group emphasizes the importance of the facility being established and operated in accordance with the principles and best practice for a global cryofacility laid out in the report.
Cryopreservation at the global banana collection at the International Transit center

Bart Panis,
Bananas
Importance of banana/plantains/cooking bananas

- Staple food for 400-1,000 million people
- Produced in >120 countries
- Banana and plantain (Musa spp.): Largest fruit crop in the world with an annual production of 129 million tonnes (2009, FAO) (Apple: 71 million tonnes)
- International banana trade: yearly turnover of ~6 billion USD
Use of bananas: Starch corm (*Ensete*)
Use of bananas; fibres (*Musa textilis*)
Use of bananas; beer bananas (East-African Highland bananas)
Use of bananas; male flower = vegetable
Use of bananas; feed
Use of bananas; flowers
Use of bananas: fruit: desert banana, snack, cooking banana (some plantains), matoke banana
cultivars
Why is conservation of the banana diversity important?
Problems: diseases

- Fungi
  - Sigatoka (*Mycosphaerella*)
  - Panama (*Fusarium*)

- Viral
  - Banana Bunchy Top Virus (BBTV)
  - Banana Streak Virus (BSV)
  - Banana Bract Mosaic Virus (BBMV)
  - Cucumber Mosaic Virus (CMV)

- Bacteria
  - Moko (*Pseudomonas*)
  - Xanthomonas

- Nematodes
  - *Radopholus similis*, *Pratylenchus goodeyi*
  - *P. coffeae*, *Helicotylenchus multicinctus*

- Insects
  - Banana weevil (*Cosmopolites sordidus*)
Other putative improvement points

- Higher production
- Better storage (shelf life)
- Fruit quality (fortification: Vitamines, Iron)
- Stressresistance
  - wind
  - salt
  - cold
  - Drought
- Banana as a biofactory (Edible vaccines)
Where do “new” cvs have to come from?

- ‘Forgotten’ cultivars

- Classical improvement (using bananas with superior characteristics)

- “Modern improvement techniques (using embryogenic cell suspensions)
How to store the banana diversity
Methods of conservation

• *In situ*: Conservation in ‘normal’ habitat
  – rain forests, gardens, farms

• *Ex Situ*:
  – Field collection, Botanical gardens
  – Seed collections
  – In vitro collection
    • Normal growth
    • Slow growth (temp↓, O₂ ↓, H₂O ↓, medium ~)
    • Cryopreservation (-196°C)

• (DNA Banks)
Bioversity International banana collection

Slow growth
- T 16°C
- PPF 25µmol.m-2.s-1 (24/24h)

Type of material
- proliferating shoot tips

Culture medium
- MS salt and vitamins, 30g.l⁻¹
  sucrose, 2.25 mg .l⁻¹ BA, 0.175 mg .l⁻¹ IAA, 2g.l⁻¹ Gelrite

Number of replicates
- 20

Storage capacity
- 12 months (4-22 months)→genotype effect!
Origin of *Musa* genetic resources

Accessions from 57 donor sources in 37 countries
## Coverage of diversity

<table>
<thead>
<tr>
<th>Holdings</th>
<th>Accessions (no.)</th>
<th>Collection (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild types</td>
<td>19 Species</td>
<td>213</td>
</tr>
<tr>
<td>Cultivated forms</td>
<td>15 groups, 40 subgroups</td>
<td>1095</td>
</tr>
<tr>
<td>Improved varieties</td>
<td>diploids, triploids, tetraploids</td>
<td>13, 33, 80</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>1434</td>
</tr>
</tbody>
</table>
Germplasm health testing


Methodologies

1/ Pre-indexing
Testing of accession lines grown in screenhouse at 3 months
Specific tests:
- PCR for BBTV/CMV/BBrMV/BSVs/BanMMV

2/ Virus therapy

<table>
<thead>
<tr>
<th></th>
<th>CMV</th>
<th>BBTV</th>
<th>BSV</th>
<th>BanMMV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Meristem culture</td>
<td>-</td>
<td>+++</td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>Thermotherapy</td>
<td>+++</td>
<td></td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>+</td>
<td>+++</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Electrotherapy</td>
<td>-</td>
<td>+++</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cryotherapy</td>
<td>+</td>
<td>+++</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

3/ VIC indexing
Testing of accession subset of TC plantlets grown in screenhouse at 3 and 6 months
Specific tests (knowns):
- Multiplex PCR for BBTV/CMV/BBrMV/BSVs
- PCR for BanMMV
- ISEM
Non-specific tests (unknowns):
- Visual inspection for symptoms
- Minipreps/EM

ITC Leuven

Pre-indexing Gembloux

Indexing VIC, Brisbane

Virus elimination Gembloux

New accession

Available for release

Not available
Musa gene bank management system (MGBMS)

- Accurate labelling of accessions (barcodes)
- Quick storage and retrieval of data (on site)
- Query and Report options available
- Flexible and simple in use
Musa Germplasm Information System (MGIS)

Holds passport data from 5000 Musa accessions maintained in 18 genebanks around the world.

By Clicking on some parts of the schema, you can access further details.
Conservation

Lyophilized tissue collection – 788 accessions

Material: leaf tissue harvested from greenhouse plants
12-20 samples of 0.5 g (fresh weight) leaf tissue
Freeze dried (48h), residual water content of 3%

Storage conditions
- Temperature: -20°C

- Side product of the rejuvenation/regeneration activity
- Low cost, simple and long term preservation of DNA
- Serving molecular scientists
Conservation of *Musa* spp. at the ITC

3 conservation methods

- Cryopreserved base collection
  - 950 accessions

- In vitro active collection
  - 1434 accessions

- Lyophilized leaf tissue collection
  - 788 accessions

Off site black box safety back-up
(IRD, France)
Cryopreservation of banana
Regenerable Musa tissues, suitable for cryopreservation

- Seed
- Zygotic embryos
- Embryogenic cell suspensions
- Somatic embryos
- Meristem cultures
2 methods for cryopreservation of banana meristems

• Droplet vitrification of ‘cauliflower like’ meristem clumps or “scalps”

• Droplet vitrification of tiny meristems (1 mm Ø) excised from *in vitro* plants
Droplet - vitrification of apical meristems

p5
3 to 7 subcultures of 5 weeks

MS+AC
1 month

1 mm apical meristem

LS
(+25 °C)
≥20 min

PVS2
(0 °C)
30 min

10 meristems in droplet PVS2 on Aluminium foil strip

liquid nitrogen (-196 °C)
> 1 h

p6
MS + 2.22 μM BA
4 to 6 weeks

MS + 0.3 M sucrose
2 days

RS
(25 °C)
15 min

Bioversity International
Lenght of PVS2 treatment
How many accessions can 1 technician cryopreserve per year?

- 50

When is an accessions considered as safely stored?

- 3 independent repetitions
- 95% certainty that at least 1 plant can be regenerated per repetition (Dussert et al., 2003)

Storage container

- 3x locator 6 plus Thermolyne (4860 tubes)
- 1x locator 4 plus Thermolyne (3240 tubes)

Black Box (at IRD, Montpellier)

- 1100 accessions (dry shipper)
Some data of 12 years cryobanking
Number of banana accessions cryopreserved
Cryopreservation is applicable to all Musa accessions
Average regeneration rates per method and per genomic group

<table>
<thead>
<tr>
<th>Method</th>
<th>Reg Scalps</th>
<th>Reg Meristems</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td></td>
<td></td>
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<tr>
<td>AAA</td>
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<tr>
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<td>AAB</td>
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<tr>
<td>AAA-h</td>
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<tr>
<td>AAB</td>
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<tr>
<td>AAB-p</td>
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<tr>
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<tr>
<td>ABB</td>
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<tr>
<td>Acuminata</td>
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<tr>
<td>balbisiana</td>
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</tr>
<tr>
<td>blanco</td>
<td></td>
<td></td>
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<tr>
<td>varia</td>
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<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
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</tr>
</tbody>
</table>
Cryopreservation of *Musa* germplasm

Bart Panis

2nd edition
Other application of cryopreservation: banana seed and embryos (wild Musa spp.)
Around 70 different wild Musa species
2 species are at the origin of edible bananas

![Map showing the distribution of species related to edible bananas.](image-url)
Germination of wild bananas is problematic!

Embryo rescue

Correlation pink embryos with red top and germination on Half MS without contamination

\[ y = 1.0105x + 1.767 \]

\[ R^2 = 0.8382 \]
Preservation average over 9 accessions

Non dry seed dies after 3 months
Dry seed can be stored at RT for at least 2 years
Best LN storage (ER)
Conclusion

- Germination of Musa seed is still problematic. How to solve this?
  - Collection time
  - Handling after harvesting
  - Germination conditions (temp, moisture, light; soil, ....)

- Wild Musa seed seems to be orthodox.

- How diverse is a population; How many seeds need to be stored to preserve the diversity
Other application of cryopreservation: 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 :
Cryopreservation methodology

- Cells 10 days after last subculture (exponential growth phase)
- Cryoprotection with 180 g/l sucrose + 7.5 % DMSO
- Slow freezing at 1°C/min to -40°C with ice-initiation at -7.5°C
- Storage in liquid nitrogen
- Rapid thawing in water bath (40°C)
- Plating on semi-solid ZZ or RD1 medium
Temperature decrease in propanol container

\[ Y = -0.97x + 26.81 \]

\[ Y = -0.43x + 7.11 \]
• After cryopreservation suspension cells need to be:

  – viable

  – able to give rise to an embryogenic cell suspension

  – true-to-type (Côte et al., 2000)

  – retain their characteristics (transformation competence)

Current situation: 2200 cryotubes with embryogenic cell lines stored in LN
Other application of cryopreservation:
Cryotherapy (viruseradication)

Viral diseases (CMV, BSV, BBTV, …..) are a constrain to banana production and to cross-border germplasm movement

A dramatic delay in the distribution of high yielding and newly bred varieties to small farmers

Eradication through

• in vitro culture
• meristem culture
• chemotherapy
• thermotherapy
• electrotherapy
• cryotherapy.
CMV and BSV infected Williams plants

In vitro cultures

CRYOPRESERVATION

Virus detection by ELISA
- 1st test on *in vitro* plants
- 2d test on *in vivo* plants
CMV ERADICATION BY CRYOPRESERVATION

bud culture (3/96)
meristem culture (0/8)
cryoprotection (1/45)
cryotherapy (24/79)

% of healthy plants

1st test
2d test
Cryotherapy (virus eradication)

- Cryopreservation act as a micro-scalpel
- Virus eradication is based on the uneven distribution of viruses in plants

IMMUNOLOCALISATION OF CMV PARTICLES (Gold-Silver Enhancement)
Other application of cryopreservation: banana nematodes

Why? For many experiments, nematode populations with different pathogenicity from different regions are needed. Out of practical reasons only 9 *Radopholus similis* populations are now stored.

Material and Methods: *Radopholus similis* populations from Ghana, Cuba, Indonesia and Uganda cultured on carrot disks or alfalfa callus. Rapid freezing in liquid nitrogen!

Results

- All populations under investigation gave rise to surviving (moving) nematodes (1-17 %)
- Surviving nematodes are able to reproduce
- Pathogenicity of cryopreserved nematodes is comparable to non-cryopreserved ones
Cryopreservation protocols are being developed for sexual as well as parthenogenetic eggs of the water flea *Daphnia magna*. Cryopreservation protocol for the larvae of the fruit fly *Drosophila melanogaster*. 
capacity building

• Over the past 25 years about 120 researchers from 44 countries were trained on plant cryopreservation techniques in Leuven. The length of their training ranged between 3 days and 1 year and they were financed by different sources (FWO, World Bank, EU, Development cooperation,….).

• We collaborated with institutes and university of 53 countries worldwide on the topic “plant cryopreservation”
Thank you

www.bioversityinternational.org