Cryobanking of Plant Genetic Resources in the Czech Republic

Cryopreservation as Safety Duplication

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1st Meeting of the ECPGR Cryopreservation Working Group
3-4 May 2023, Crop Research Institute, Prague, Czech Republic
The National Programme on Conservation and Utilization of Plant Genetic Resources and Agrobiodiversity

- Organized by Ministry of Agriculture
- Coordinated by the Crop Research Institute
- Board of plant genetic resources - curators of generatively and vegetatively propagated crops
  - Generatively propagated crops (cereals, ..) stored in form of seed at low temperature for few or tens years in the Central Seed Genebank
  - Vegetatively propagated crops – storing in form of seeds is not possible, stored in vegetatively propagated part of plants – tubers, bulbs, cuttings, ex vitro explants or intact plants in field conditions; backup in the Central Cryobank
The National Programme on Conservation and Utilization of Plant Genetic Resources and Agrobiodiversity

**Vegetatively propagated crops - National curators:**

- Potato research Institute Havlíčkův Brod – potato (*in vitro*)
- Hop Research Institute Žatec – hop
- MENDELU Lednice – thermophilic temperate fluit trees
- CRI Olomouc – *Allium*
- VSV Karlštejn CRI, Ampelos Vrbovec, MENDELU Lednice – *Vitis*
- Research and Breeding Institute of Pomology Holovousy – temperate fruit trees
Vegetatively propagated crops - National curators:

Basic strategy of plant germplasm cryoconservation

– safety duplication of basic collections (different storage method and locality)

– storing the most valuable genetic material of the Czech origin

Central cryobank in the frame of „National program“ – collaborates with plant germplasm curators, that provide the most valuable samples for their backup.

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## Current Cryopreservation Activities

**Cryobank - current state**

<table>
<thead>
<tr>
<th>Crop number</th>
<th>Crop code</th>
<th>Crop name</th>
<th>Number of accessions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F01</td>
<td><em>Malus domestica</em> BORK</td>
<td>17</td>
</tr>
<tr>
<td>2</td>
<td>F07</td>
<td><em>Pyrus communis</em> L. (E</td>
<td>24</td>
</tr>
<tr>
<td>3</td>
<td>F24</td>
<td><em>Prunus armeniaca</em> L.</td>
<td>12</td>
</tr>
<tr>
<td>4</td>
<td>F28</td>
<td><em>Persica vulgaris</em> P.M</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>F35</td>
<td><em>Cerasus avium</em> (L.) M</td>
<td>3</td>
</tr>
<tr>
<td>6</td>
<td>F37</td>
<td><em>Cerasus vulgaris</em> P.M</td>
<td>10</td>
</tr>
<tr>
<td>7</td>
<td>F38</td>
<td><em>Cerasus P.MILLER</em> (ot</td>
<td>3</td>
</tr>
<tr>
<td>8</td>
<td>F46</td>
<td><em>Fragaria x ananassa</em></td>
<td>34</td>
</tr>
<tr>
<td>9</td>
<td>F80</td>
<td><em>Lonicer L. (edible</em></td>
<td>24</td>
</tr>
<tr>
<td>10</td>
<td>H01</td>
<td><em>Allium sativum</em> L.</td>
<td>187</td>
</tr>
<tr>
<td>11</td>
<td>S01</td>
<td><em>Solanum tuberosum</em> L1</td>
<td>104</td>
</tr>
<tr>
<td>12</td>
<td>V01</td>
<td><em>Vitis vinifera</em> L.</td>
<td>3</td>
</tr>
<tr>
<td>13</td>
<td>W93</td>
<td>*Malus MILL. &lt;short. c</td>
<td>6</td>
</tr>
<tr>
<td>14</td>
<td>X90</td>
<td><em>Humulus lupulus</em> L.</td>
<td>68</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td></td>
<td><strong>500</strong></td>
</tr>
</tbody>
</table>
Current Cryopreservation activities

**FUNDING**

- Institutional project: 22%
- National projects: 32%
- International project: 37%
- National program: 9% (0.7 personal capacity)
Current Cryopreservation activities

Tripartite German-Czech-Polan *Allium* cryobank - preservation of valuable accessions of garlic gene pools on the basis of mutual reciprocity within the framework of tripartite international cooperation, which is the result of a joint GENRES research project called EURALLIVEG (Jiri Zamecnik)

“Healthy berries in a changing climate: development of new biotechnological procedures for virus diagnostics, vector studies, elimination and safe preservation of strawberry and raspberry” – international cooperation project Czech Rep. + Norway (NIBIO) (Alois Bilavcik)

“Nanocomposite hydrogels for cryopreservation of plant genetic resources“ within the programme Horizon Europe, call “MSCA4Ukraine” - Grant Agreement No. 1233650 (Olena Bobrova)

Genotyping-by-sequencing of the European garlic collection to develop a sustainable ex situ conservation strategy (Garli-CCS) - Sixth Call, Phase X, ECPGR Grant
Cryopreservation protocols

- **plant material** – *ex vitro*, *in vitro*

- **acclimation** – low temperature, osmotic

- **methods** - two-step freezing, encapsulation-dehydration, simple-dehydration, vitrification, droplet-vitrification

- **recovery** - safe cryopreservation and recovery of samples
Cryopreservation protocols

- Two-step freezing – dehydration by freezing
- Encapsulation-dehydration – dehydration by dry air
- Simple-dehydration – dehydration by dry air
- Vitrification – osmotic dehydration
- Droplet-vitrification – osmotic dehydration
Cryopreservation protocols

- **Two-step freezing** – dehydration by freezing
- Encapsulation-dehydration
- Simple-dehydration
- Vitrification
- Droplet-vitrification
Two-step cryopreservation

1. step

- Dehydration
- Equilibration
- Liquid nitrogen

2. step

- Rehydration
- Regeneration

1st Meeting of the ECPGR Cryopreservation Working Group
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Cryopreservation protocols

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Two-step cryopreservation

I. step
- Dehydration
- Equilibration
- Liquid nitrogen

II. step
- 2 °C: 6 h
- 1000 °C: 3 h
- 690 °C: 0 h

Regeneration
- Visual evaluation – browning of tissues, sprouting in water or peat, grafting
- Chip budding – orchard, glasshouse
- The end of May - beginning of July
- Evaluation – after 100 – 130 days

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Cryopreservation protocols

• Two-step freezing
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**Cryopreservation protocols**

- **Two-step freezing**
- **Encapsulation-dehydration**
- **Simple dehydration**
- **Vitrification**
- **Droplet-vitrification**

**1st Meeting of the ECPGR Cryopreservation Working Group**

**Sampling of buds**

- Orchards - **ecodormant**
- Temperature – longer period of **subzero temperature**
- Keeping – temperature – 4 °C

**Pretreatment of samples**

- Cutting
- Dehydration & cold hardening
- **Thermal characteristics determination (DSC, DTA)**

**Warming and rehydration**

- Warming - at 4 °C
- Rehydration – moist white peat at 4 °C

**Regeneration**

- Visual evaluation – browning of tissues, sprouting in water or peat, grafting
- Chip budding – orchard, glasshouse
- **The end of May - beginning of July**
- Evaluation – after 100 – 130 days

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Cryopreservation protocols

- Two-step freezing

- Encapsulation-dehydration – dehydration by dry air

- Simple-dehydration – dehydration by dry air

- Vitrification

- Droplet-vitrification
Dissection and encapsulation of *in vitro* cultures

- Two-step freezing
- Encapsulation-dehydration
- Simple dehydration
- Vitrification
- Droplet-vitrification

Encapsulated shoot tip

Cryopreservation protocols

- Two-step freezing
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Dehydration

Encapsulated shoot tips
Regeneration of encapsulated shoot tips

Regenerating plants (30 days after thawing)

Regrowing shoot tip (14 days after warming)
Cryopreservation protocols

- Vitrification
- Droplet-vitrification

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Cryopreservation protocols

Recovery - safe cryopreservation and recovery of samples
Cryopreservation protocols

**Recovery** - safe cryopreservation and recovery of samples

**Minimal number of stored samples**
- Number of stored samples – 120 shoot tips, 20 pcs for control recovery
- Minimal explant regeneration – 20 -30 %

**Stefan Dussert probability tool**
- Minimal number of stored shoot tips – 120 pcs
- Minimal size of control sample – 40 pcs
- Minimal explants recovery – 30%
- Minimal number of recovered shoot tips from total amount stored – 14 pcs
Cryotherapy

Virus elimination by cryopreservation

• Potato

• Hop

• Garlic

• Raspberry
## Cryotherapy

### POTATO

<table>
<thead>
<tr>
<th>Method</th>
<th>PLRV</th>
<th>PVY</th>
<th>PVS</th>
</tr>
</thead>
<tbody>
<tr>
<td>thermontherapy</td>
<td>28%</td>
<td>24%</td>
<td>0%</td>
</tr>
<tr>
<td>chemotherapy</td>
<td>0%</td>
<td>22% *</td>
<td>80%</td>
</tr>
<tr>
<td>cryotherapy</td>
<td>67%</td>
<td>64%</td>
<td>0%</td>
</tr>
</tbody>
</table>

* Not succesfull for PVY- O

### HOPS

<table>
<thead>
<tr>
<th>Method</th>
<th>ApMV</th>
<th>HMV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thermontherapy</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>Cryotherapy</td>
<td>15%</td>
<td>88%</td>
</tr>
</tbody>
</table>

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Thermal analysis as a tool for cryopreservation protocol development

Thermal Analysis – Differential Scanning Calorimetry

- **heat flow measurement** during temperature change – **assessment of heat capacity changes** – connected with changes of a state of matter – liquid vs solid, crystals vs glassy state

- the first-order transition events – **crystallization or melting** (connected with transition energy release) the second-order transition event – **glassy state**

- **freezable water content**
Thermal analysis as a tool for cryopreservation protocol development

- Thermal Analysis – Differential Scanning Calorimetry
  - heat flow measurement during temperature change
  - assessment of heat capacity changes connected with changes of a state of matter – liquid vs solid, crystals vs glassy state
  - the first-order transition events – crystallization or melting (connected with transition energy release)
  - the second-order transition event –
  - freezable water content

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- Thermal Analysis – Differential Scanning Calorimetry
  - Heat flow measurement during temperature change
  - Assessment of heat capacity changes connected with changes of a state of matter – liquid vs solid, crystals vs glassy state
  - The first-order transition events – crystallization or melting (connected with transition energy release)
  - The second-order transition event – glassy state
- Freezable water content

- The first-order transition events
- The second-order transition events
- Freezable water content
Thermal analysis as a tool for cryopreservation protocol development

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- heat flow measurement during temperature change
- assessment of heat capacity changes connected with changes of a state of matter – liquid vs solid, crystals vs glassy state

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**VITRIFICATION CONTROL BY DSC AT 10 °C/min.**

<table>
<thead>
<tr>
<th>CPA conc. group</th>
<th>SOLUTE CONCENTRATION (g / g)</th>
<th>CRYOPRESERVATION CONDITIONS</th>
<th>CRITICAL COOLING RATE</th>
<th>CRITICAL WARMING RATE</th>
<th>FREEZING/MELTING during cooling / warming</th>
<th>GLASSY STATE</th>
<th>WATER CONTENT</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0–0.5</td>
<td>Near-equilibrium freezing</td>
<td>CCR&gt;10 °C/min.</td>
<td>T_h / T_m</td>
<td>T_g ≈ T_g(MFCP)</td>
<td>&gt;1</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.5–0.6</td>
<td>Supercooling</td>
<td>CCR&lt;10 °C/min.</td>
<td>− / T_m</td>
<td>T_g</td>
<td>1–0.67</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.6</td>
<td>– devitrification sensitive</td>
<td>CWR≤10 °C/min.</td>
<td>− / − (T_m)</td>
<td>T_g</td>
<td>0.67</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>0.7</td>
<td>Vitrification</td>
<td>CWR&lt;10 °C/min.</td>
<td>− / −</td>
<td>T_g</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.8</td>
<td>– „stable“</td>
<td>CWR~0 °C/min.</td>
<td>− / −</td>
<td>T_g ≈ T_g(MFCP)</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>&gt;0.8</td>
<td>Supersaturated solution</td>
<td>CCR&gt;10 °C/min.</td>
<td>T_h / T_m</td>
<td>T_g ′</td>
<td>&lt;0.25</td>
<td></td>
</tr>
</tbody>
</table>

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Prospects and limits of cryobanking

Goals:
• Improving knowledge of cryotolerance
• Development of protocols for sensitive plant species and genotypes
• Complete the cryopreservation of selected types of crops of national importance
• Health status control of explants
• Sharing information about cryobanking
• Cooperation on international projects

Limits:
• unstable and insufficient funding of the cryobank
Thank you for your attention!

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