

SHORT-TERM SCIENTIFIC MISSION, COST863

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SCIENTIFIC REPORT

The application and the elaboration of the protocol for encapsulation-dehydration, vitrification and droplet vitrification techniques in raspberry (*Rubus idaeus* L.)

Introduction

Fruit Tree Research Centre of Rome is the repository of the *in vivo* national collection of the fruit tree germplasm with about 8,000 genotypes (Sartori et al., 2003). Furthermore, properly equipped *in vitro* culture laboratory in the Propagation section and trained staff in the cryopreservation techniques (Damiano et al., in press) are also present for high quality training.

Over the past decades, plant cryopreservation technologies have been evolving rapidly, opening the door to the long-term storage of valuable genetic resources of many crop species (Panis and Lambardi, 2005). Thus, from the original slow-cooling approach, research has moved to easier and more reproducible techniques which allow a complete vitrification of extra- and intracellular liquids through the direct immersion of explants in liquid nitrogen (LN).

The term cryopreservation refers to the storage of specimens in liquid nitrogen, at ultra-low temperature (-196°C). Firstly, the cryogenic technique was mainly based on slowcooling methods in which optimal cell dehydration is achieved by a controlled freezing rate of explants to about 40°C before the immersion in LN (Lambardi and De Carlo, 2003).

Cryopreservation of biological tissue can be successful only if intra-cellular ice crystal formation is avoided since this causes irreversible damage to cell membranes, destroying their semi-permeability. According to Panis and Lambardi (2005), in nature, some plant species adopted systems where the ice crystal formation at sub-zero temperatures can be avoided through the synthesis of specific substances (such as sugars, proline and proteins) that lower the freezing point in the living plant cells resulting in „supercooling“. But crystal formation without an extreme reduction of cellular water can be only prevented through vitrification.

Vitrification is the second approach to cryopreservation and involves pretreatment of germplasm with high-concentrated chemical cryoprotectant mixtures (Reed et al., 2001). Panis and Lambardi (2005) showed that the method of droplet vitrification, a modification of the vitrification method, applied to *Musaceae* gave higher post-thaw regeneration rates than regular cryovial freezing protocol.

The third approach to plant cryopreservation is encapsulation-dehydration (Dereuddre et al., 1990). Plant tissues are encapsulated in alginate beads, exposed to osmotic and dehydrated to a critical moisture level.

In the last years the CRA – FRU of Rome has introduced these three cryopreservation methods and therefore by courtesy of the hosts I applied these methods to raspberry during STSM.

Material and Methods

In vitro propagated raspberry *Rubus idaeus* L., cv Latham, was used as a plant source. ‘Latham’ is an old cultivar. It is suitable for intermountain regions, though the incidence of mildew can be a problem in highly humid areas. A slow warming spring is required for optimal flower and fruit production.

Two type of explants were used, i.e. shoot tips from *in vitro* plants grown under normal conditions (24°C) and from cold storage at (+5°C, 4 months) (Fig. 1, 2)



Fig. 1. Cv Latham grown at 24°C



Fig. 2. Cv Latham grown at 5°C

1. Encapsulation - Dehydration

The method of Dereuddre et al. (1990) was used. After the dissection, 2-3 mm long apices were partially immersed into petri dishes containing a multiplication medium to avoid drying out. The explants were subsequently immersed into a 3% alginic acid sodium salt medium. Using a cut-tip propipette, beads were sucked out and transferred to the medium supplemented with CaCl_2 where they were kept for 30 minutes (Fig. 3). Encapsulated apices were pregrown for the different periods of time (1 to 3 d) in a liquid medium, sucrose concentration ranging from 0.75 M and 1M.

Dessication was performed by placing the beads in small sterilized containers (80°C over night in a dryer) with silica gel for 2 or 8 hrs (Fig. 4). The moisture rate for each material was calculated for each treatment.

After the dessication, beads were transferred into 2 ml sterile cryovials and immersed immediately in LN for 1h. Thawing was performed by placing the cryotubes in the air current of the laminar flow cabinet for 2 min (Fig. 5). Beads were then transferred in Petri dishes containing standard multiplication medium and were kept in darkness for one week where they were cultured under standard conditions in growth room.

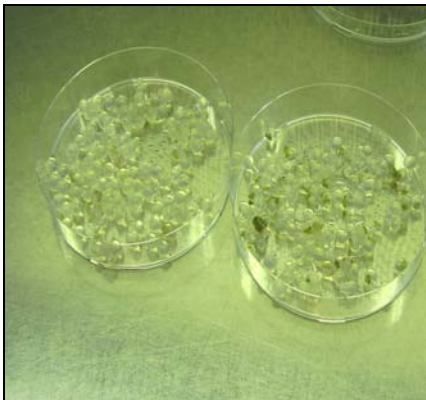


Fig 3. Alginate beads

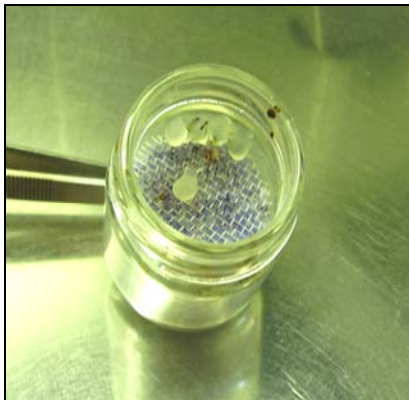


Fig. 4. Dessication

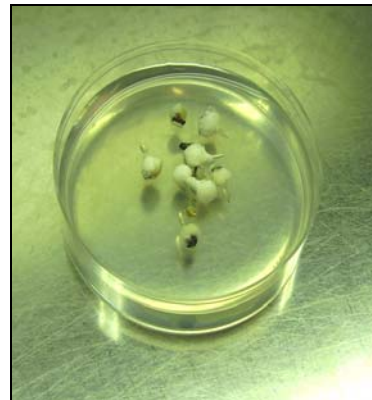


Fig. 5. Beads after LN treatment

2. Vitrification/one-step freezing

The key step in this procedure is the pretreatment of explants with highly concentrated vitrification solutions which induce dehydration of cells.

Dessicated shoot tips (2-3 mm) in LAF were put in cryovials of 2 ml supplemented with loading solution (LS) (MS, sucrose, glycerol) for 20 min.

The LS solution was then extracted with Paster's pipette and 2 ml PVS2 (Plant Vitrification Solution n° 2) was added, as firstly proposed by Sakai et al. (1991). The solution is a mixture of cryoprotectants, i.e. 30% glycerol, 15% ethylene glycol and 15% dimethylsulfoxide (DMSO) in MS liquid medium supplemented with 0.4 M sucrose. Explants were kept for 30 minutes on ice in PVS2 solution (Fig. 6), then they were immersed in LN for 10 minutes and thawed in water bath at 40°C for 1.5 min. Upon eliminating PVS2, 2 ml of Recovery Solution (RS: MS, plus 0.3 M sucrose) was added and kept for 15 min.

After this step, RS was taken off with Paster's pipette and explants were transferred into Petri dishes containing a hormone-free multiplication medium supplemented with 0.3 M sucrose (Fig. 7). Eventually, they were taken to Growth Room (GR) where they were kept in darkness for one day.

The following day, the explants were transferred onto the multiplication medium supplemented with 2.22 μ M BA and returned to GR where they were maintained for 7 days. Regrowth was monitored after that period.



Fig. 6. Explants in PVS2



Fig. 7. Medium with 0.3 M sucrose

3. Droplet vitrification

This technique is relatively new and so far has been applied to only a limited number of species. The technique was introduced recently in the CRA-FRU through a co-operation with dr. Panis of the University of Lovain.

Excised explants were placed into LS for 20 minutes, LS was then poured off with Paster's pipette and PVS2 was added and explants were kept on ice for 20 minutes.

Petri dishes, in which thin foil pieces were lying, were placed on ice bag (Fig. 8). The control was introduced directly into RS and it was shaken occasionally (15 min). Explants were then placed onto the tin foil pieces covered with a drop of PVS2 whereupon the thin foil pieces were immersed with forceps into LN for ten minutes. After 15 minutes, the control was transferred into Petri dishes containing the RS medium supplemented with 0.3 M sucrose. Tin foil pieces were taken out from LN with forceps and transferred into Petri dishes containing the RS medium where they were kept for 15 minutes. The dishes should be shaken occasionally. Explants were then transferred to Petri dishes containing the RS medium supplemented with 0.3 M sucrose and were subsequently moved to growth room for 7 days. Regrowth will be determined upon the expiry of the period.

Conclusion

The set objective for this STSM has been fully completed. The protocols for these three methods have been mastered and the samples have been brought along to monitor the regrowth. The established cooperation will be continued along through the material and scientific information exchange with joint work on cryopreservation of fruit species.

This experience provides good grounds for the establishing of a cryopreservation laboratory and for the initiation of the work on collecting most valuable small fruit germplasm in Serbia.

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