Cryopreservation of cherry rootstock Gisela 5 using vitrification procedure

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Abstract

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In vitro-grown shoot tips of Gisela 5 (*Prunus cerasus* × *Prunus canescens*) cherry rootstock were tested for regrowth after cryopreservation using vitrification technique. Explants were precultured in the dark at 23°C, in a liquid MS medium with a progressively increasing sucrose concentration (0.3 M for 15 h, then 0.7 M for 5 h), and subsequently loaded in a solution containing 2 M glycerol and 0.4 M sucrose for 20 minutes. Shoot tips were dehydrated at 0°C using either the original PVS2 or modified PVS2 solution (PVS A3 – 22.5% sucrose, 37.5% glycerol, 15% ethylene glycol and 15% DMSO) for 30, 40 and 50 minutes. The survival and regrowth of the cryopreserved shoot tips dehydrated with the original PVS2 solution ranged between 36–54% and 8–17%, respectively. However, the dehydration with the PVS A3 solution resulted in considerably higher survival rates (81–92%), as well as higher regrowth rates (39–56%) after cryopreservation. These results prove the feasibility of the PVS A3-based vitrification technique for a long-term storage of this genotype.

Keywords: *Prunus cerasus* × *Prunus canescens*; liquid nitrogen; vitrification solutions

It is estimated that up to 100,000 plants representing more than one third of the world's total plant species are currently threatened or face extinction (Panis, Lambardi 2005). According to IPGRI (2004, in: Panis, Lambardi 2005), 6 million samples of plant genetic resources are held in national, regional, international and private gene bank collections around the world.

Advances in biotechnology, especially in the area of *in vitro* culture techniques, provide some important tools for improved conservation and management of plant genetic resources.

The advantages of cryopreservation are that germplasm can be kept for a theoretically indefinite time, at a low cost and taking up very little space (Kaviani 2011).

Cryopreservation is becoming a very important tool for long-term storage of plant germplasm, favouring high genetic stability and minimal maintenance requirements. Cryopreservation, i.e. the storage of biological material at ultralow temperature, usually that of liquid nitrogen (LN, –196°C) is the only technique currently available to ensure safe and cost-efficient long-term conservation of different types of germplasm (Engelmann 2004).

Among other cryotechniques, cryopreservation by shoot-tip vitrification is very promising for long-term storage of fruit species (De Carlo et al. 2000). Vitrification refers to the physical process by which a concentrated aqueous solution solidifies into a metastable glass in the tissue, at sufficiently low temperatures, without the occurrence of ice

crystallization (Engelmann 1991; Shatnawi et al. 2007).

Cryopreservation of biological tissues can be successful only if intra-cellular ice crystal formation is avoided, since this causes irreversible damage to cell membranes, thus destroying their semi-permeability (Panis, Lambardi 2005). Crystal formation without an extreme reduction of cellular water can only be prevented through vitrification. Vitrification/one-step freezing relies on a treatment of the explants with a concentrated vitrification solution for variable periods of time, from 15 min to up to two hours, followed by a direct plunge into LN (Panis, Lambardi 2005). Vitrification-based methods involve the removal of most or all of freezable water by physical or osmotic dehydration of explants, followed by ultra-rapid freezing which results in vitrification of intracellular solutes, i.e. formation of an amorphous glassy structure without the occurrence of ice crystals which are detrimental to cellular structure integrity (Kaviani 2011).

The aim of the present study was to develop a cryopreservation protocol for *in vitro* shoot tips of Gisela 5 rootstock based on the vitrification procedure.

MATERIAL AND METHODS

Preculture (pregrowth) of isolated shoot tips. Apical shoot tips, 2 mm in length, were dissected under the binocular and placed on a solid Murashige and Skoog (1962) (MS) medium containing 1 mg/l N^6-benzyladenine (BA), 0.1 mg/l indole-3-butyric acid (IBA) and 0.1 mg/l gibberellic acid (GA_3) with 0.3 M sucrose and 7 g/l agar (pH 5.7) (Vujović et al. 2012).

After dissection, isolated shoot tips were placed in Erlenmeyer flask filled with the medium for preculture I (pregrowth medium I): liquid MS medium of the same hormonal composition with 0.3 M sucrose. The flask was covered with aluminium foil and placed in the growth room over the night (15 h). After that period, explants were transferred in the Erlenmeyer flask and poured over with the medium for preculture II (pregrowth medium II): liquid MS medium with 0.7 M sucrose, covered with aluminium foil and placed in the growth room for a 5-h incubation. After the preculture, 10 shoot tips were transferred directly onto the filter paper placed in sterile Petri dishes on the semisolid, hormone-free MS medium with 0.3 M sucrose and 3 g/l agar, pH 5.7, and left in the growth room for 1 day. After that, the explants were transferred onto the regrowth medium (solified MS medium with 20 g/l sucrose and 7 g/l agar), covered with aluminium foil and placed in the growth room for 7 days, before being transferred to the standard conditions (at 23 ± 1°C, 16 h day/8 h night photoperiod and 8.83 W/m^2 light intensity on culture surface) – marked as 'pregrowth control'.

Loading treatment. After pregrowth treatment II, shoot tips were placed on dry and sterile filter paper and then transferred in filter-sterilized loading solution poured in cryovials (10 explants per cryovial). The loading solution contained 2 M glycerol and 0.4 M sucrose dissolved in liquid, hormone-free MS medium (pH 5.7). The excised tips were left in the loading solution for 20 min at room temperature, in the dark (covered with aluminium foil).

After the loading treatment, 10 shoot tips were transferred directly into the recovery (unloading) solution for 15 min at room temperature, in the dark, dried on sterile filter paper and subsequently transferred on the regrowth medium. Recovery (unloading) solution contained 1.2 M sucrose dissolved in liquid, hormone-free MS medium (pH 5.7). Petri dishes with explants covered with aluminium foil were placed in the growth room as described previously. These explants were designated as 'loading controls'.

Vitrification. After the loading treatment, the loading solution was replaced with 2 ml of filter sterilised vitrification solution. Vitrification (dehydration) was performed on ice (0°C) using 2 types of vitrification solutions: PVS2 vitrification solution (Sakai et al. 1990) (liquid, hormone-free MS medium containing in w/v, 13.7% sucrose, 30% glycerol, 15% ethylene glycol and 15% dimethylsulfoxide – DMSO) and modified PVS2 vitrification solution – PVS A3 solution (Kim et al. 2009) (liquid, hormone-free MS medium containing in w/v: 22.5% sucrose, 37.5% glycerol, 15% ethylene glycol and 15% DMSO). The following durations of dehydration were used for both types of vitrification solutions: 30, 40 and 50 minutes. After dehydration, ten explants per each treatment (type of vitrification solution and duration of dehydration) were directly transferred to the recovery (unloading) solution (without freezing in LN), incubated for 15 min at room temperature, in the dark, and subsequently transferred on the regrowth medium as described previously – marked as 'vitrification or dehydration controls' (–LN).

Cryopreservation. At the end of the vitrification treatment, cryovials containing explants were directly plunged in LN and kept for at least 10 minutes. Approximately twenty shoot tips (2 repetitions with 10 explants per each repetition) were used for each type of vitrification solution and treatment duration. For fast rewarming, cryovials were retrieved from LN and plunged into a water bath at 40°C for 1.5 min, after which the vitrification solution was removed and replaced with the recovery (unloading) solution for further incubation (15 min at room temperature). After rewarming, shoot tips were retrieved from the unloading solution, dried, transferred on sterile filter paper placed on the semisolid MS medium with 0.3 M sucrose and 3 g/l agar, pH 5.7, poured in sterile Petri dishes covered with aluminium foil and placed in growth room for 1 day. Following this, the explants were transferred onto the regrowth medium (solid MS medium for multiplication with 20 g/l sucrose and 7 g/l agar), covered with aluminium foil, placed in the growth room for 7 days and then transferred to standard conditions as it was previously described.

Survival and regrowth. Survival was evaluated 2 weeks following the cryopreservation (1 week-growth in dark + 1 week-growth under standard conditions) by counting the number of shoots that showed any sign of growth (formation of callus, appearance of viable/green structures as a sign of a beginning of regeneration), while regrowth was defined as further development of apices into shoots with developed leaves, 8 weeks after rewarming. Also, the number of regenerants per shoot tip was counted.

Multiplication monitoring in successive subcultures after regeneration. After regrowth, shoots were subcultured in two successive subcultures on MS medium supplemented with 1 mg/l BA, 0.1 mg/l IBA, 0.1 mg/l GA_3, 20 g/l sucrose and 7 g/l agar. The duration of the subculture was 21 days and the following multiplication parameters were monitored: multiplication index, length of axial and lateral shoots. Multiplication index was defined as the mean number of newly formed shoots (> 0.5 cm) per initial shoot recorded after the stated subculture interval. Due to the low multiplication index in the second subculture, shoots were placed in the third subculture on the MS medium with only BA at 3.37 mg/l, sucrose 20 g/l and agar 7 g/l (very good results obtained with Gisela 6 rootstock, unpublished data).

Statistical analysis. All data were analysed by ANOVA, followed by the Duncan's Multiple Range Test for $P < 0.05$. Data presented in the form of percentage were subjected to arcsine transformation.

RESULTS AND DISCUSSION

This experiment evaluated the survival and regeneration rates, as well as multiplication parameters of three successive subcultures of Gisela 5 shoot tip explants after rewarming from storage in LN.

In the vitrification method, cells and shoot tips must be sufficiently dehydrated by the vitrification solution (which hardly penetrates into cells during the dehydration process) without causing injury, in order to be able to vitrify upon rapid cooling in LN (Sakai, Engelmann 2007). Thus the key for successful cryopreservation by vitrification is to quantify dehydration tolerance of the samples to be cryopreserved to the PVS2 solution (Sakai, Engelmann 2007) or some other type of vitrification solution.

In a vitrification protocol, tolerance to vitrification solutions is acquired by optimizing the preconditioning and loading treatments, as well as the duration and temperature of exposure to the vitrification solution. The type of vitrification solutions used in our experiment (PVS2 and PVS A3) and the immersion time in them showed statistical differences (Fig. 1). Pregrowth control and loading control showed the highest percentages of survival and regrowth, as well as the non-cryopreserved explants, i.e the so-called vitrification or dehydration controls (–LN) (Fig. 1a).

Preculture or pregrowth involves preculturing the germplasm on a medium supplemented with cryoprotectants such as sucrose or glucose before its exposure to LN (Kaviani 2011). For many species, preculture with sucrose appears to be insufficient to produce a high level of survival by vitrification. Thus samples have to be treated with a cryoprotective solution (loading solution), with an intermediate concentration to prepare them for exposure to the vitrification solution (Sakai, Engelmann 2007). A mixture of 2 M glycerol (18%) plus 0.4 M sucrose (14%) was very effective in inducing tolerance to dehydration by the vitrification solution. This loading solution proved to be satisfactory also for Gisela 5 explants, because apart from the 100% survival of pregrowth control explants and loading control explants, the first signs of regeneration were observed after two weeks from their

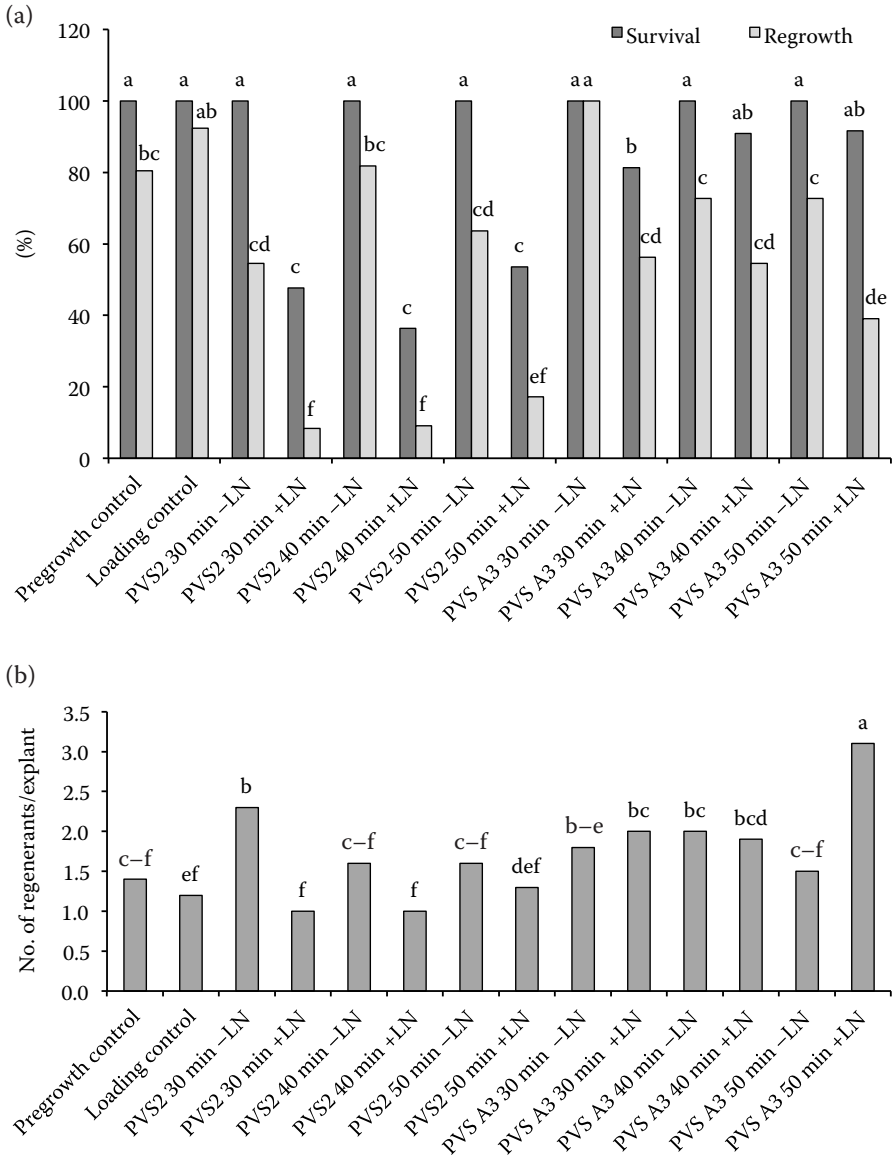

Fig. 1. Survival and regrowth (a) and regenerated shoots per explant (b) of control (–LN) and cryopreserved explants (+LN) dehydrated with PVS2 and PVS A3 vitrification solutions on ice

mean values followed by the same letter are not significantly different according to the Duncan's Multiple Range test ($P < 0.05$)

placement on the regeneration medium, with morphologically normal shoots with formed 1–2 leaves (Fig. 2). Kaviani (2011) showed that osmoprotection with 2 M glycerol and 0.4 M sucrose is effective in enhancing the capacity of cells to tolerate dehydration with PVS2.

It is well-known that in using the PVS2 vitrification solution (Sakai et al. 1990) the selection of proper incubation time is a key step for obtaining high percentages of explant survival after direct plunging into LN and thawing. Thus immersion in PVS A3 gave the highest survival rate of Gisela 5 explants being 92% for a 50-min treatment, and also 91%, for the 40-min treatment. The best result with PVS2 vitrification solution was also obtained with the 50-min treatment, but being considerably lower – 54% (Fig. 1a). Similar regrowth percentages were obtained with PVS A3 vitrification solution applied for 30 and 40 min, being 56% and 54% respectively. The beginning of regeneration of cryopreserved explants dehydrated with PVS A3 was noticed after 3 weeks and up to week 8 the shoots were well developed with a lot of light-green leaves without any signs of chlorosis (Fig. 3), which was detected on the shoots dehydrated with PVS2 for 30 and 40 minutes.

The survival rate of the vitrified shoot tips of cherry cultivar Sendaiya increased gradually with the duration of exposure to PVS2 and reached the max. value after 90 and 105 min of exposure (Niino et al. 1997). We observed similar increasing survival trend, especially with PVS A3. The incubation time with vitrification solutions appears to be species-specific.

Table 1. Multiplication parameters of shoots regenerated from control explants and cryopreserved explants in three successive subcultures after regrowth

No. of treatment	Multiplication index			Length of axial shoot (cm)			Length of lateral shoots (cm)			Average No. of buds < 0.5 cm/axial shoot		
	1. subc. A	2. subc. A	3. subc. B	1. subc. A	2. subc. A	3. subc. B	1. subc. A	2. subc. A	3. subc. B	1. subc. A	2. subc. A	3. subc. B
1	1.00 ns	1.09^{bc}	1.33^{a}	0.54^{defg}	0.97^{bc}	1.22^{a}	–	0.60^{ab}	0.70^{a}	0^{c}	0^{c}	4.00^{a}
2	1.10 ns	1.30^{a}	1.12^{cd}	0.84^{ab}	0.91^{cde}	0.93^{bcd}	–	0.52^{bc}	0.50^{b}	0^{c}	1.40^{a}	2.44^{ef}
3	1.00 ns	1.07^{bc}	1.00^{e}	0.68^{bcd}	1.07^{ab}	1.07^{ab}	–	0.60^{ab}	–	0^{c}	0^{c}	3.22^{abcde}
4	1.00 ns	1.25^{ab}	1.00^{e}	0.40^{g}	0.58^{h}	0.74^{d}	–	0.50^{c}	–	1.25^{a}	0^{c}	2.90^{cdef}
5	1.00 ns	1.38^{a}	1.00^{e}	0.64^{cde}	0.86^{cde}	0.88^{bcd}	–	0.64^{a}	–	0^{c}	0^{c}	3.56^{abc}
6	1.00 ns	1.25^{ab}	1.25^{ab}	0.42^{fg}	0.65^{gh}	0.84^{cd}	–	0.50^{c}	0.50^{b}	0^{c}	0^{c}	3.42^{abcd}
7	1.00 ns	1.20^{ab}	1.08^{de}	0.88^{a}	1.12^{a}	1.07^{ab}	–	0.50^{c}	0.50^{b}	0^{c}	0^{c}	2.64^{def}
8	1.00 ns	1.00^{c}	1.18^{bc}	0.48^{efg}	0.80^{ef}	1.00^{bc}	–	–	0.50^{b}	1.00^{b}	0^{c}	2.95^{bcdef}
9	1.00 ns	1.00^{c}	1.00^{e}	0.76^{abc}	0.81^{def}	1.02^{abc}	–	–	–	0^{c}	0^{c}	3.22^{abcde}
10	1.00 ns	1.07^{bc}	1.00^{e}	0.48^{efg}	0.77^{efg}	0.90^{bcd}	–	0.50^{c}	–	0^{c}	1.28^{ab}	2.34^{ef}
11	1.00 ns	1.11^{bc}	1.00^{e}	0.62^{cde}	0.72^{fgh}	0.91^{bcd}	–	0.50^{c}	–	0^{c}	0^{c}	2.13^{f}
12	1.00 ns	1.00^{c}	1.10^{d}	0.60^{cdef}	0.95^{bcd}	1.06^{ab}	–	–	0.50^{b}	0^{c}	1.36^{a}	3.80^{ab}
13	1.00 ns	1.08^{bc}	1.00^{e}	0.70^{abcd}	0.86^{cde}	1.03^{abc}	–	0.50^{c}	–	0^{c}	0^{c}	2.66^{def}
14	1.00 ns	1.08^{bc}	1.00^{e}	0.70^{abcd}	0.82^{def}	1.22^{a}	–	0.50^{c}	–	0^{c}	1.00^{b}	3.84^{a}

mean values of multiplication parameters within each column followed by the same letter are not significantly different according to the Duncan's Multiple Range Test ($P < 0.05$); ns – non significantly; A – shoots cultivated on MS medium with BA 1, IBA 0.1 and GA_3 0.1 mg/l; B – shoots cultivated on MS medium with 3.37 mg/l BA

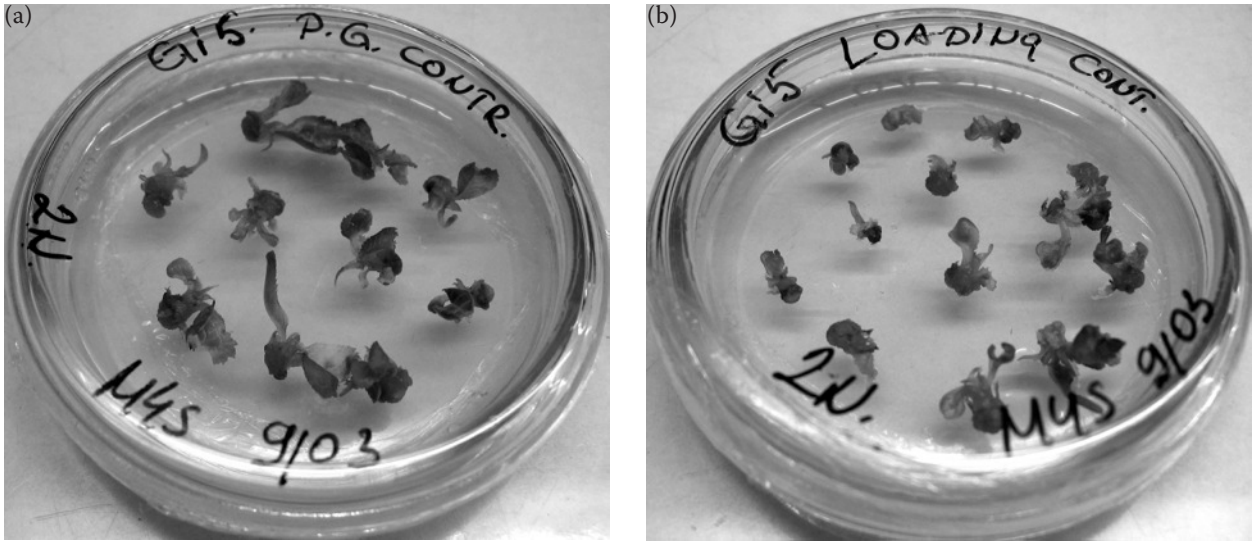

Fig. 2. Survival of control explants, 2 weeks after placement on regeneration medium: (a) pregrowth control and (b) loading control

According to Shatnawi et al. (2007), exposure to the vitrification solutions decreased the regrowth of non-frozen shoot tips which was observed only in the treatment with PVS A3 in our case (Fig. 1a).

However, the percentage of shoot formation of unfrozen shoot tips was generally higher than that of cryopreserved tips. The survival of the control (non-frozen) embryoaxes of *Citrus madurensis* (Gi Cho et al. 2002) ranged between 63–98% depending on the loading solution employed, while the values obtained in our trial were much higher, reaching up to 100% with both vitrification solutions (Table 1). The percentage of shoot formation after cryopreservation of *Prunus avium* ranged from 8.6–77.8% (Shatnawi et al. 2007), but in our trial with Gisela 5 rootstock the regrowth ranged from 8–56% and was the best with PVS A3, 30 min incubation (Fig. 1a).

It is important to observe that when explants are loaded with the PVS2 solution, the post-thaw survival is to a great extent influenced by the duration of the treatment, which must be long enough to ensure sufficient cell dehydration without cytotoxic effects (Panis, Lambardi 2005).

The protective effect of the loading treatment might be a result of the cryopreservation of cyto-

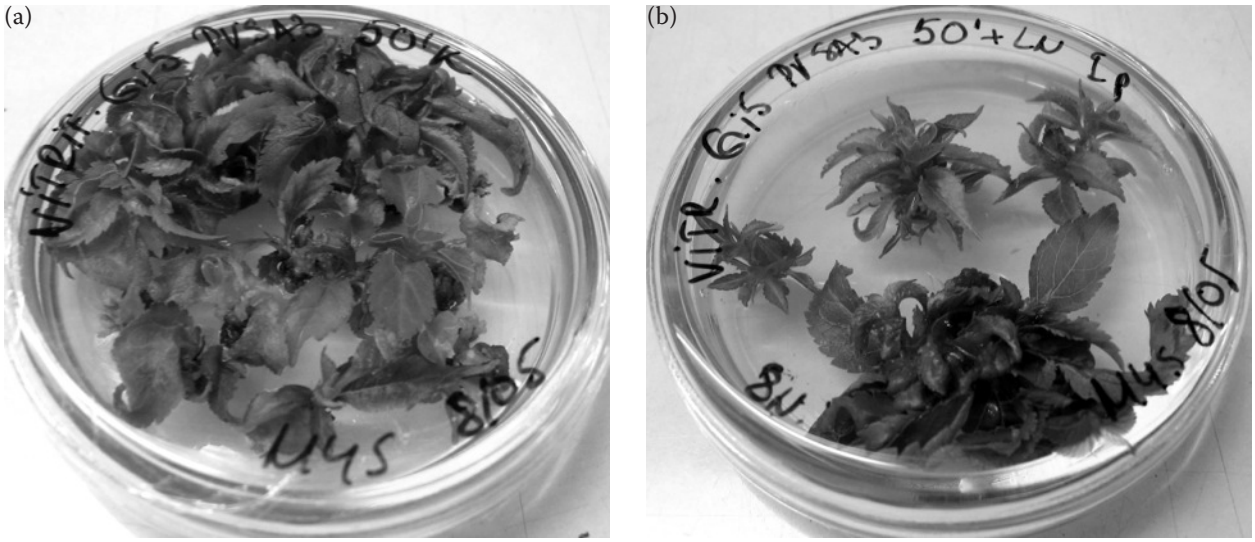

Fig. 3. Regrowth of explants dehydrated 50 min with PVS A3 on ice, 8 weeks after placement on regeneration medium: (a) control explants (non-cryopreserved, –LN) and (b) cryopreserved explants (+LN)

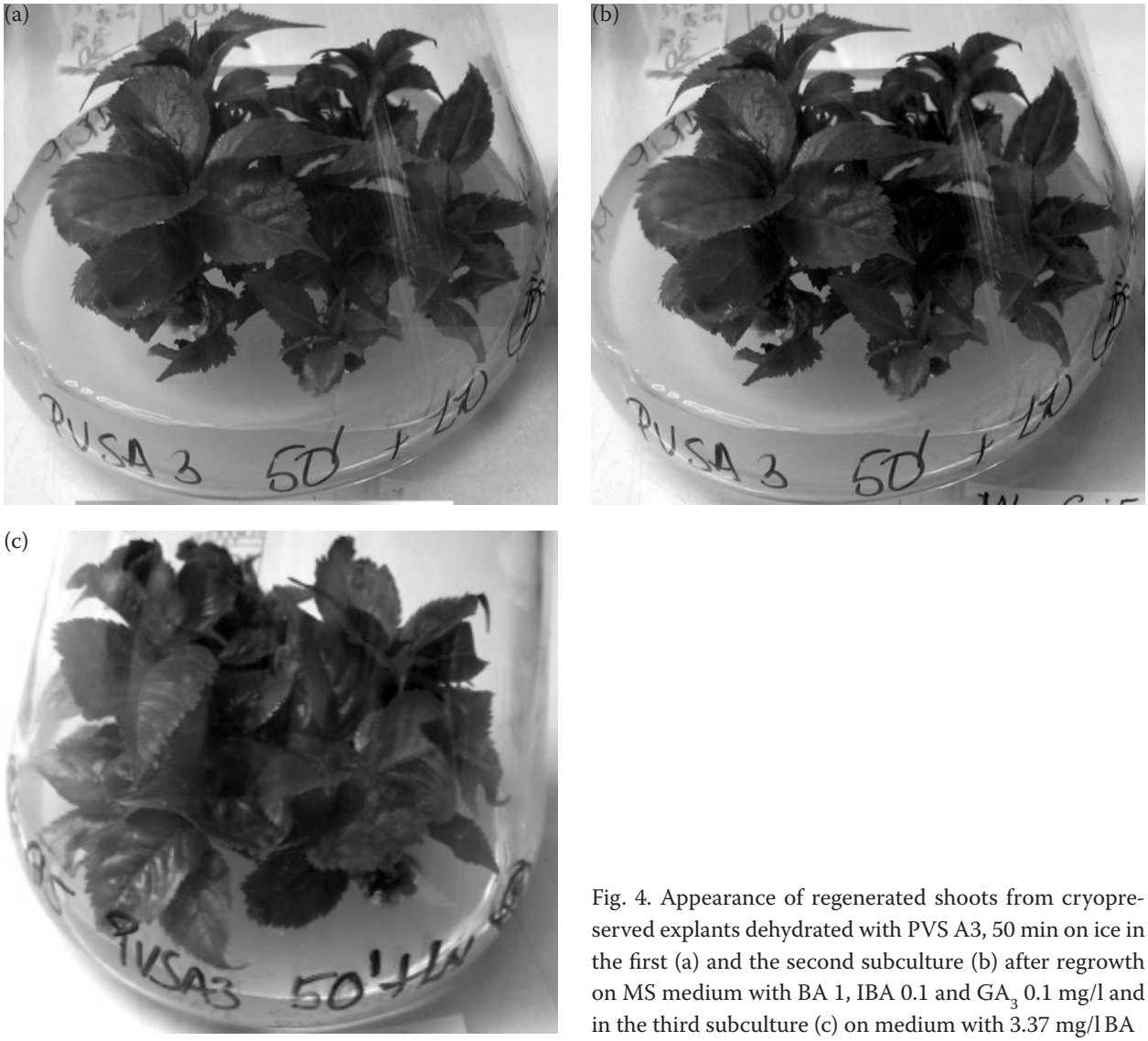

Fig. 4. Appearance of regenerated shoots from cryopreserved explants dehydrated with PVS A3, 50 min on ice in the first (a) and the second subculture (b) after regrowth on MS medium with BA 1, IBA 0.1 and GA_3 0.1 mg/l and in the third subculture (c) on medium with 3.37 mg/l BA

solic cryoprotectants accumulated during the preculture with sucrose and of the protective effects of plasmolysis (Sakai et al. 1990). Rapid warming in a waterbath is required to avoid recrystallization and ensures a proper recovery of the vitrified material (Panis, Lambardi 2005), with 40°C as the most commonly applied temperature.

The crucial factor for the evaluation of cryopreservation methods is regeneration, because only regeneration of whole plants from cryopreserved shoot tips may be recognized as a success of the method (Kryszczuk et al. 2006). The success of cryopreservation depends on various factors, such as the cryopreservation method and the plant genotypes. Regeneration rates for Gisela 5 rootstock shoot tips cryopreserved by droplet vitrification technique were higher with the PVS3 vitrification solution applied in the 60-min incubation (40%), but similar to PVS A3 (37%) over an incubation time of 50 min (unpublished data), in comparison with cryopreservation by vitrification technique. The success of the procedure can be attributed to its ease, high reproducibility and to the fact that it can be successfully applied to a wide range of tissues and plant species (Panis, Lambardi 2005).

It is very important to know which processes occur during cryopreservation and thus affect the success of cryomethods. According to Kaviani (2011) at cryo temperature (–196°C) cell division, metabolic and biochemical activities remain suspended and the material can be stored without changes and deterioration for a long time. Ultrastructural changes during cryopreservation are important to understand and improve this method (Kaviani 2011). The plasma membrane is considered to be one of the most important determinants

for survival at low temperatures. During the process of cryopreservation, the plasma membrane is subjected to severe, multiple stresses due to various treatments which are prerequisite for survival (Uemura et al. 2009). They reported that both lipid and protein composition of the plasma membrane alter dynamically during cold acclimation, which ultimately results in an increase in the cryostability of the plasma membrane.

The cryoprotective substances are also important. The most common cryoprotective substances are DMSO, polyethylene glycol (PEG), sucrose, sorbitol and mannitol. These substances have the osmotic actions. However, some of them – such as DMSO – can enter cells and protect cellular integrity during cryopreservation (Kaviani 2011).

The success of a cryopreservation method also depends on the tolerance and sensitivity of plant germplasm to the stresses of the cryopreservation method (Reed et al. 2005).

To expedite the implementation of cryopreservation protocols in large-scale international gene banks it is essential to develop technically simple and robust protocols that may be applied across a wide genetic basis. This implementation is closely related to the shoot multiplication after cryopreservation. In our experiment the better values of multiplication parameters were obtained in the third subculture after regrowth, with the cryopreserved shoots dehydrated with PVS A3 vitrification solution, especially with the 50 min incubation (Table 1), without signs of chlorosis in all three subcultures (Fig. 4).

This work has contributed to the opportunities of wider application leading toward standardizing and simplifying the cryomethods.

CONCLUSION

The best survival and regrowth after cryopreservation of Gisela 5 rootstock explants were obtained by dehydration with PVS A3 vitrification solution for all the investigated incubation times (30, 40 and 50 min).

After three successive subcultures, multiplication of this genotype was established, with a normal shoot morphology.

The experiment indicates that the final results may be improved by modifications of the cryopreservation protocol, including other types of vitrification solutions and incubation time, because the key of successful cryopreservation using vitrification is to achieve tolerance to dehydration by using a highly concentrated vitrification solution which can be expected to enable significant shoot formation after freezing in LN.

The future research will focus on further optimization of the cryogenic protocols to expand the application for a wide range of fruit species.

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