Journal of Horticultural Research 2013, vol. 21(2): 79-85
DOI: 10.2478/johr-2013-0025

CRYOPRESERVATION OF CHERRY ROOTSTOCK GISELA 5 (*PRUNUS CERASUS* × *PRUNUS CANESCENS*) SHOOT TIPS BY DROPLET-VITRIFICATION TECHNIQUE

Djurdjina RUŽIĆ*[1], Tatjana VUJOVIĆ[1], Radosav CEROVIĆ[2]
[1]Fruit Research Institute, Kralja Petra I/9, 32000 Čačak, Republic of Serbia
[2]Innovation Center, Faculty of Technology and Metallurgy,
Karnegijeva 4, 11120 Belgrade, Republic of Serbia

Received: November 2, 2013; Accepted: November 20, 2013

ABSTRACT

The droplet-vitrification technique was applied to *in vitro* shoot tips of cherry rootstock Gisela 5 (*Prunus cerasus* × *Prunus canescens*). Explants were precultured in the dark at 23 °C, in liquid MS medium with a progressively increasing sucrose concentration (0.3 M for 15 h, then 0.7 M for 5 h). Loading involved a 30 min incubation of explants in a solution comprising 1.9 M glycerol and 0.5 M sucrose. Explants were dehydrated at room temperature using a solution PVS A3 [Murashige and Skoog (MS) liquid medium, 22.5% (w/v) sucrose, 37.5% (w/v) glycerol, 15% (w/v) ethylene glycol and 15% (w/v) dimethylsulfoxide] for 30, 40 and 50 min and the PVS3 solution [MS liquid medium, 50% (w/v) sucrose, 50% (w/v) glycerol] for 60, 90 and 120 min. Explants were cooled by direct immersion in liquid nitrogen (LN) in 10 µl droplets of vitrification solution placed on aluminum foil strips. The foil strips were retrieved from LN and immersed in preheated (37 °C) unloading solution (0.8 M sucrose) for 30 s, and an equal volume of unloading solution at room temperature was added for further incubation for 30 min. Shoot tips were transferred onto the regrowth medium, cultivated in the dark for 7 days before being incubated under standard conditions.

Three weeks after transferring the shoot tips onto the regrowth medium, the survival rate of control and cryopreserved explants of Gisela 5 dehydrated with PVS A3 was 100%, regardless of the treatment duration. After dehydration with solution PVS3, the survival varied between 70 and 100% for control explants and 78 and 95% for cryopreserved shoot tips. Gisela 5 shoot tips dehydrated for 40 min with PVS A3 vitrification solution demonstrated the best regrowth (38%). When using the PVS3 solution, survival of cryopreserved shoot tips was the highest (95%) after 60 min treatment followed by 40% regrowth.

After three successive subcultures on shoot multiplication, medium shoots recovered viability, multiplication ability and morphology equal of that prior to cryopreservation.

Key words: cherry rootstock, cryopreservation, droplet-vitrification, vitrification solutions

INTRODUCTION

Plant gene banks are maintained mainly *in situ* in the field. These collections are suitable for breeding, but impose high requirements in terms of space (land) and costs. Moreover, collections are exposed to biotic stress (excessive diseases as well as high pest pressure) and abiotic external stress factors. Therefore, establishment of a modern germplasm collection of fruit plants necessarily involves the use of *in vitro* techniques as an important supplement to conventional preservation of germplasm in the field (Reed et al. 2008).

Over the recent decades, cryopreservation has become a very important tool for long-term conservation of plant germplasm. Nevertheless, a wider application of plant cryopreservation depends on the availability of efficient, reproducible and robust

*Corresponding author:
e-mail: djinaruzic@gmail.com

cryopreservation protocols applicable to different plant species (Panis & Lambardi 2005). Among the currently available cryotechniques droplet-vitrification has so far been applied to a limited number of plant species (Sakai & Engelmann 2007), especially fruit species such as *Musaceae* (Panis et al. 2005), blackberry and cherry plum (Vujović et al. 2011), apple and raspberry (Condello et al. 2011a, b). The droplet method was first reported by Schäfer-Menuhr et al. (1994) using potato apices.

Although several vitrification solutions (VSs) have been developed for cryopreserving plant tissues, the most commonly used and most efficient are the glycerol-based vitrification solutions PVS2 (Sakai et al. 1990) and PVS3 (Nishizawa et al. 1993). However, dehydration procedures need to be carefully controlled to prevent biochemical and/or osmotic toxicity of cryoprotectant solutions. Recently, Kim et al. (2009b) developed alternative VSs, modified from the original PVS2 or PVS3 by increasing or decreasing the concentration of the constitutive cryoprotectants, which could be successfully employed with plant materials that were very sensitive to these highly toxic VSs (Kim et al. 2009b).

This paper presents the results of the application of the droplet-vitrification technique for cryopreservation of *in vitro* grown shoot tips of cherry rootstock Gisela 5. The droplet-vitrification protocol was performed by evaluating the effect of two vitrification solutions and different treatment durations on recovery of explants after the liquid nitrogen (LN) exposure.

The aim of this paper was to develop a standard protocol for Gisela 5 conservation to be used for formation of national *in vitro* fruit gene bank.

MATERIALS AND METHODS

Plant material

In vitro grown shoot tips of cherry rootstock Gisela 5 (*Prunus cerasus* × *Prunus canescens*) were tested for regrowth after cryopreservation. Aseptic culture of this species had previously been established and multiplied at the Tissue Culture Laboratory of Fruit Research Institute, Čačak (Republic of Serbia). Individual shoot tips (1-2 mm in length) were dissected under a binocular microscope, transferred on solidified Murashige and Skoog (Murashige & Skoog 1962) MS medium supplemented with 1 $mg \cdot l^{-1}$ N6-benzyladenine (BA), 0.1 $mg \cdot l^{-1}$ indole-3-butyric acid (IBA), 0.1 $mg \cdot l^{-1}$ gibberellic acid (GA_3), and 0.3 M sucrose and 7 $g \cdot l^{-1}$ agar (pH 5.7), and kept in the dark for as long as the isolation lasted.

Cryopreservation technique employed

Immediately after isolation, shoot tips were precultured in the dark at 23 °C, in liquid MS medium with a progressively increasing sucrose concentration (0.3 M for 15 h, then 0.7 M for 5 h). Following this, 10 explants were directly transferred onto the multiplication medium (solidified MS medium with 1 $mg \cdot l^{-1}$ BA, 0.1 $mg \cdot l^{-1}$ IBA, 0.1 $mg \cdot l^{-1}$ GA_3, 20 $g \cdot l^{-1}$ sucrose and 7 $g \cdot l^{-1}$ agar), and left in a dark growth room for 7 days. Thereafter, the explants' growth was continued in standard conditions (16 h daylight/8 h dark). This explant's treatment was defined as the pre-growth control.

Loading involved 30 min incubation of explants in a solution comprising 1.9 M glycerol and 0.5 M sucrose (Kim et al. 2009a). After the loading, 10 explants were directly transferred into the unloading solution made of liquid hormone-free MS medium with 0.8 M sucrose (Kim et al. 2009b), where they were kept for 30 min before being placed on the regrowth medium. This explant/treatment combination was defined as the loading control.

The next step was dehydration performed at room temperature using two types of VSs: PVS3 vitrification solution (liquid hormone-free MS medium with in w/v: 50% sucrose, 50% glycerol) and modified PVS2 vitrification solution (PVS A3) (Kim et al. 2009b) (liquid hormone-free MS medium with in w/v: 22.5% sucrose, 37.5% glycerol, 15% ethylene glycol and 15% dimethylsulfoxide, DMSO). PVS3 vitrification solution was applied for 60, 90 and 120 min, whereas PVS A3 vitrification solution was applied for 30, 40 and 50 min. The treatment duration was chosen based on previous study conducted with cherry plum and blackberry (Vujović et al. 2011).

Explants were cooled by direct immersion in liquid nitrogen (+LN) in 10 µl droplets of vitrification solution placed on aluminum foil strips, and kept for at least 30 min. The foil strips were retrieved from LN and immersed in a preheated (37 °C) unloading solution (0.8 M sucrose) for 30 s, before adding an equal volume of unloading solution at room temperature for further 30 min incubation (Kim et al. 2009b). Dehydration controls refer to replicates carried out under the same conditions as cryopreservation experiments but without immersion in LN (-LN).

Shoot tips were transferred onto the re-growth medium, cultivated in the dark for 7 days, before being transferred to standard conditions. The survival rate was evaluated 3 weeks after cryopreservation by counting the number of explants that showed any kind of growth (callus formation, appearance of viable/green structures, as an indication of the beginning of regeneration).

Regeneration, that is, re-growth, was defined as further development of apices into shoots with developed leaves 4-8 weeks after re-warming. The number of regenerants per explants was also determined.

Growth recovery after cryopreservation

Following the re-growth, the shoots were subcultured twice on the MS multiplication medium with 1 $mg \cdot l^{-1}$ BAP, 0.1 $mg \cdot l^{-1}$ IBA, 0.1 $mg \cdot l^{-1}$ GA_3, 20 $g \cdot l^{-1}$ sucrose and 7 g l^{-1} agar. Each subculture lasted 21 days, after which period the following multiplication parameters were determined: multiplication index, length of axial and lateral shoots. Since no significant multiplication was observed after two subcultures, in the third subculture the shoots were placed on the MS medium with 3.37 $mg \cdot l^{-1}$ BA (no other plant growth regulators), which proved highly efficient in the Gisela 5 cherry rootstock multiplication (unpublished data).

Statistical analysis of results

The experiments were repeated twice and 10-15 shoot tips were used per treatment. All the obtained data were statistically analysed using the Analysis of Variance (ANOVA) and then Duncan's Multiple Range Test for $P < 0.05$. Data presented in the form of percentage were subjected to the arcsine transformation.

RESULTS

The explants defined as the pre-growth control in this experiment showed the first signs of regrowth as early as 2 weeks after they had been placed on the regeneration medium. The morphological appearance of the shoots was typical, with one to two leaves. After 4 weeks, shoots were fully formed with a number of green leaves (Fig. 1A). The beginning of growth of explants dehydrated using PVS3 or PVS A3 but not treated with LN was detected 3 weeks after their placement on the regeneration medium. However, these explants demonstrated slower growth, so that the shoots of the explants dehydrated using PVS A3 were not fully formed until 4-6 weeks after placement on the regeneration medium, while those of explants dehydrated using PVS3 were formed 7-8 weeks after placement on the medium.

The beginning of the re-growth in the cryopreserved explants dehydrated using both VSs was also detected 3 weeks following placement onto the regeneration medium. After 8 weeks, growth of shoots derived from the explants that were dehydrated for 40 min in PVS A3 was most advanced (Fig. 1B). Among shoots treated with PVS3 and cryopreserved, the largest shoots were obtained from the explants dehydrated for 60 min (Fig. 1C), while the increase in dehydration time using this solution led to reduced quality of regenerated shoots that tended to become smaller in size and even show signs of chlorosis (Fig. 1D).

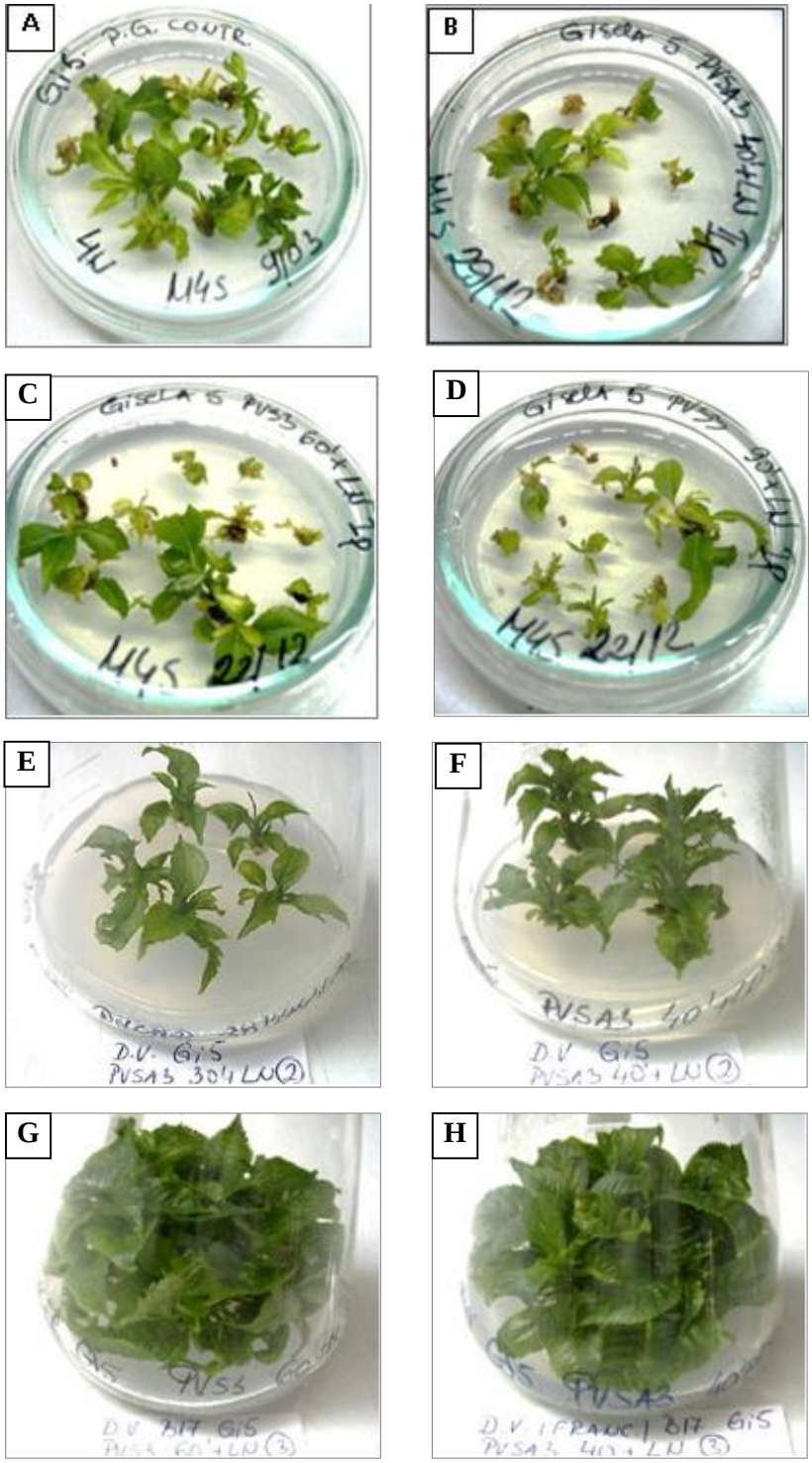

Fig. 1. Regrowth of Gisela 5 explants. Pre-growth control after 4 weeks (A); cryopreserved explants dehydrated for 40 min with PVS A3 after 8 weeks (B); cryopreserved explants dehydrated for 60 min with PVS3 after 8 weeks (C); cryopreserved explants dehydrated for 90 min with PVS3 after 8 weeks (D); control explants dehydrated with PVS A3 for 30 min in the second subculture after re-growth on MS medium with BA 1, IBA 0.1 and GA_3 0.1 $mg \cdot l^{-1}$ (E); cryopreserved explants dehydrated with PVS A3 for 40 min in the second subculture after re-growth on MS medium with BA 1, IBA 0.1 and GA_3 0.1 $mg \cdot l^{-1}$ (F); cryopreserved explants dehydrated with PVS3 for 60 min in the third subculture after re-growth on MS medium with 3.37 $mg \cdot l^{-1}$ BA (G); cryopreserved explants dehydrated with PVS A3 for 40 min in the third subculture after re-growth on MS medium with 3.37 $mg \cdot l^{-1}$ BA (H)

The survival rate of both control and cryopreserved explants of Gisela 5 dehydrated with PVS A3 amounted to 100%, regardless of the treatment duration (Table 1). However, after dehydration with solution PVS3, the survival significantly varied between 70 and 100% for control explants and 78 and 95% for cryopreserved shoot tips, with the highest values being achieved with the shortest treatment duration in both type of explants. On the other hand, the type of vitrification solution and duration of treatment did not significantly affect re-growth of cryopreserved shoot tips, which varied between 19 and 38% for PVS A3 treatments and between 26 and 40% for PVS3 treatments. The highest percentage of re-growth was obtained using PVS3 for 60 and 90 min and PVS A3 for 40 min (Table 1).

At the beginning of subculturing, the shoots that were regenerated from non-cryopreserved and cryopreserved explants dehydrated with both PVS A3 and PVS3 were short, with a small habit and small leaves: traits especially present in explants dehydrated using PVS A3 (Fig. 1E). However, the control and cryopreserved shoots failed to multiply in the first and the second subculture on a medium containing 1 $mg \cdot l^{-1}$ BA, 0.1 $mg \cdot l^{-1}$ IBA and 0.1 $mg \cdot l^{-1}$ GA_3 (Table 2) (Fig. 1F). In the third subculture, all of the shoots on the medium with the increased concentration of BA (3.37 $mg \cdot l^{-1}$), regardless of their origin, demonstrated a good appearance, with a long stem and sizeable habit, as well as wide green leaves and no signs of chlorosis (Fig. 1G, H). Although in this subculture a low multiplication index was recorded, most of the shoots (regardless of their origin) formed a large number of buds at the base of the shoots, 0.3-0.4 cm long. The highest multiplication index in the third subculture was obtained in shoots originated from explants dehydrated with PVS A3 for 40 min and PVS3 for 90 min (Table 2). The 60 min treatment with PVS3 produced the highest quality plantlets after cryopreservation (Fig. 1G). Although the rate of multiplication did not exceed two shoots, their quality was very good for further subculturing.

Table 1. Survival and regrowth of control (-LN) and cryopreserved explants (+LN) dehydrated with PVS3 and PVS A3 vitrification solutions at room temperature

Treatment	Survival (%)	Regrowth (%)	No regenerants/ explant
Pregrowth control	100.00 a[1]	80.46 a	1.4 a
Loading control	100.00 a	60.00 ab	1.2 a
PVS A3 30 min -LN	100.00 a	36.36 bcd	1.5 a
PVS A3 30 min +LN	100.00 a	19.09 d	1.0 a
PVS A3 40 min -LN	100.00 a	50.00 bc	1.4 a
PVS A3 40 min +LN	100.00 a	38.18 bcd	1.2 a
PVS A3 50 min -LN	100.00 a	27.27 cd	1.2 a
PVS A3 50 min +LN	100.00 a	37.27 bcd	1.0 a
PVS3 60 min -LN	100.00 a	36.36 bcd	1.0 a
PVS3 60 min +LN	95.00 ab	40.00 bcd	1.2 a
PVS3 90 min -LN	70.00 d	60.00 ab	1.2 a
PVS3 90 min +LN	90.46 bc	38.09 bcd	1.1 a
PVS3 120 min -LN	81.82 cd	18.18 d	1.0 a
PVS3 120 min +LN	77.78 cd	26.11 cd	1.0 a

[1]Mean values within same column followed by the same letter are not significantly different according to Duncan's Multiple Range Test ($P \leq 0.05$).

Table 2. Shoot multiplication parameters of control and cryopreserved explants in three successive subcultures after regrowth. Subcultures 1 and 2 on the medium with BA 1, IBA 0.1 and GA_3 0.1 mg·l^{-1}; subculture 3 on the medium with BA 3.37 mg·l^{-1}

Treatment/min	Multiplication index			Length of axial shoot (cm)			Length of lateral shoots (cm)		
	1.	2.	3.	1.	2.	3.	1.	2.	3.
Pregrowth control	1.00	1.09	1.33 ab[1]	0.54 ab	0.97 a	1.22 bcde	-	0.60	0.70a
Loading control	1.00	1.00	1.25 bc	0.48 abc	0.58 def	1.15 cde	-	-	0.50d
PVS A3/30 – LN[a]	1.00	1.00	1.18 cd	0.56 a	0.62 de	1.23 bcde	-	-	0.50 d
PVS A3/30 + LN[b]	1.00	1.00	1.10 de	0.40 c	0.45 g	1.21 bcde	-	-	0.50 d
PVS A3/40 - LN	1.00	1.00	1.43 a	0.41 c	0.57 def	1.13 e	-	-	0.53 cd
PVS A3/40 + LN	1.00	1.00	1.36 ab	0.41 c	0.65 cd	1.32 b	-	-	0.50 d
PVS A3/50 - LN	1.00	1.00	1.25 bc	0.43 bc	0.51 fg	1.29 bcd	-	-	0.50 d
PVS A3/50 + LN	1.00	1.12	1.25 bc	0.40 c	0.74 bc	1.10 e	-	0.5	0.55 bcd
PVS3 /60 - LN	1.00	1.00	1.00 e	0.45 abc	0.59 def	1.14 de	-	-	-
PVS3/60 + LN	1.00	1.00	1.17 cd	0.42 bc	0.54 efg	1.48 a	-	-	0.50 d
PVS3/90 - LN	1.00	1.00	1.40 a	0.48 abc	0.77 b	1.31 bc	-	-	0.62 ab
PVS3/90 + LN	1.00	1.10	1.36 ab	0.39 c	0.59 def	1.11 e	-	0.50	0.60 bc
PVS3/120 - LN	1.00	1.00	1.00 e	0.40 c	0.45 g	0.77 f	-	-	-
PVS3/120 + LN	1.00	1.00	1.00 e	0.45 abc	0.50 fg	1.09 e	-	-	-
	ns[2]	ns	$P \leq 0.05$	$P \leq 0.05$	$P \leq 0.05$	$P \leq 0.05$	-	ns	$P \leq 0.05$

[1] Mean values within same column followed by the same letter are not significantly different according to Duncan's Multiple Range Test ($P \leq 0.05$)

[2] ns – not significant; [a]without cryopreservation; [b]cryopreserved

DISCUSSION

A quantification of the dehydration tolerance of a species to vitrification solutions is important for the effect of cryopreservation (Sakai & Engelmann 2007). The influence of VSs is usually highly species-specific. Vujović et al. (2011) demonstrated that the re-growth percentages achieved and the qualitative characteristics of re-growing shoots were lower in cherry plum explants than in blackberry. Similarly, the duration of dehydration is equally important and has to be determined very precisely in order to find a correct balance between toxicity and adequate dehydration so that the possibility of ice crystal formation in cryopreserved tissues is reduced (Panis et al. 2005).

According to Vujović et al. (2011) re-growth of blackberry was more effective using PVS3 than modified PVS2 solution, while the type of VS and the duration of treatment did not significantly affect the re-growth of cherry plum shoot tips which was also observed in our study with cherry rootstock Gisela 5. As regards treatments duration, our study revealed that similar to blackberry, shorter PVS3 treatments increase the re-growth of cryopreserved shoot tips. By contrast, prolonged dehydration with PVS A3 (but within shorter time borders) improved re-growth of cherry rootstock Gisela 5. A similar effect of prolonged PVS A3 treatment duration was noticed with blackberry where 30 min treatment resulted in a significant increase in regeneration percentage (30%), as compared with a 10 min treatment with the same solution (5%) (Vujović et al. 2011). According to Sakai et al. (2008), the response is related to the species and to the size of the explants.

To improve the re-growth percentage, it is important to find the critical steps of this cryo method. Thus, according to Reed et al. (2008), the protocol for cryopreservation of *Rubus* germplasm using PVS2-based vitrification should include 2-10 weeks of cold acclimation to acquire tolerance to PVS2.

Although the application of PVS3 is limited due to its tendency to induce high osmotic stress (Kim et al. 2009b), in our experiments Gisela 5 explants withstood a broad range of treatments with

PVS3 (up to 90 min) without any significant decrease in re-growth percentage. However, good results were also obtained with 40 min PVS A3 treatment.

Based on the obtained results, droplet-vitrification is a promising cryopreservation method for Gisela 5 rootstock. Further optimization of the protocol may be achieved by decreasing/ increasing the duration of treatment with vitrification solutions or employing other PVS2-based solutions. Since pretreatment and loading control resulted in very good survival and re-growth percentages, it is necessary to indicate the step(s) of the protocol of most importance for the success of this cryopreservation method. In addition, optimization of media for multiplication of cryopreserved shoots is a necessary measure to be taken, as an important precondition for the preservation of the germplasm in *in vitro* conditions.

Acknowledgement

This investigation was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia through the project No. TR-31064.

REFERENCES

Condello E., Caboni E., Andrè E., Piette B., Druart P., Swennen R., Panis B. 2011a. Cryopreservation of apple *in vitro* axillary buds of using droplet–vitrification. Cryoletters 32: 175-185.

Condello E., Ružić D., Panis B., Caboni E. 2011b. Raspberry cryopreservation by droplet vitrification technique. Proc. of XXVIII International Horticultural Congress on Science and Horticulture for People (IHC2010): III International Symposium on Plant Genetic Resources, Acta Hort. 918: 965-969.

Kim H.H., Lee Y.G., Park S.U., Lee S.C., Baek H.J., Cho E.G., Engelmann F. 2009a. Development of alternative loading solutions in droplet-vitrification procedures. Cryoletters 30: 291-299.

Kim H.H., Lee Y.G., Shin D.J., Ko H.C., Gwag J.G., Cho E.G., Engelmann F. 2009b. Development of alternative plant vitrification solutions in droplet-vitrification procedures Cryoletters 30: 320-334.

Murashige T., Skoog F. 1962. A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol. Plant. 15: 473-497.

Nishizawa S., Sakai A., Amano Y., Matsuzawa T. 1993. Cryopreservation of asparagus (*Asparagus officinalis* L.) embriogenetic suspension cells and subsequent plant regeneration by vitrification. Plant Sci. 91: 67-73.

Panis B., Lambardi M. 2005. Status of cryopreservation technologies in plants (crops and forest trees). Proc. International Workshop 'The Role of Biotechnology for the Characterisation and Conservation of Crop, Forestry, Animal and Fishery Genetic Resources', Turin, Italy 5-7 March 2005, pp. 43-54.

Panis B., Piette B., Swennen R. 2005. Droplet vitrification of apical meristems: a cryopreservation protocol applicable to all *Musaceae*. Plant Sci. 168: 45-55. DOI: 10.1016/j.plantsci.2004.07.022

Reed B., Hummer K., Gupta S., Chang Y. 2008. Medium and long-term storage of *Rubus* germplasm. Proc. IX Inter. *Rubus* and *Ribes* Symposium, Acta Hort. 777: 91-97.

Sakai A., Engelmann F. 2007. Vitrification, encapsulation-vitrification and droplet-vitrification: a review. Cryoletters 28(3): 151-172.

Sakai A., Kobayashi S., Oiyama I. 1990. Cryopreservation of nucellar cells of navel orange (*Citrus sinensis* Osb. Var. *brasiliensis* Tanaka) by vitrification. Plant Cell Rep. 9: 30-33. http://dx.doi.org/10.1007/BF00232130.

Sakai A., Hirai D., Takao N. 2008. Development of PVS-based vitrification and encapsulation-vitrification protocols. In: Reed B.M. (Ed.), Plant cryopreservation: A practical guide, Springer, USA, pp. 33-57. http://dx.doi.org/10.1007/978-0-387-72276-4_3.

Schäfer-Menuhr A., Schumacher H.M., Mix-Wagner G. 1994. Long-term storage of old potato varieties by cryopreservation of shoot-tips in liquid-nitrogen Landbauforschung Völkenrode 44 (4): 301-313 [in German with English abstract].

Vujović T., Sylvestre I., Ružić Đ., Engelmann F. 2011. Droplet-vitrification of apical shoot tips of *Rubus fruticosus* L. and *Prunus cerasifera* Ehrh. Sci. Hortic. 130: 222-228. DOI: 10.1016/j.scienta.2011.06.049.