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Thermal analysis of grapevine shoot tips during dehydration and vitrification

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Summary

The wider use of cryopreservation methods for conservation of plant genetic resources is limited by a sensitivity of different genotypes to specific procedures. Thermal analysis can improve routine use of these methods, by means of determination of melting point and proportion of freezable water after dehydration at each step of the cryoprotocol. Changes in these characteristics during explants dehydration were compared with changes in their viability in three grapevine varieties ('Blussard modry', 'Portugal modry' and 'Kerner'). The highest sensitivity to dehydration was found in the variety 'Kerner'. The proportion of freezable water during dehydration was affected mostly by cryoprotective solutions and partly by genotype. Tested protocol could allow using the cryopreservation procedure for preservation of grapevine variety with different sensitivity to dehydration. It was demonstrated that thermal analysis was a useful tool to validate cryoprotective solutions used for grapevine cryoconservation.

K e y w o r d s : cryoprotectants; dehydration; DSC; plant vitrification solution; *Vitis*.

Introduction

Cryopreservation is a method for long-term preservation of living cells and tissues at liquid nitrogen temperature. This method can be used for the conservation of plant genetic resources and thus eliminate the risk of their losses due to biotic or abiotic stressors. Different cryoprotocols has been recently developed for a wide range of different crops. Utilization of these methods can be limited by staff experience, specific conditions in different labs or variability of plant material. Although, a small number of cryopreservation methods were developed for grapevine cryoconservation, their utilization is still limited. Cryopreservation of grapevine began in the early 90s of the last century (Plessis *et al.* 1991, Dussert *et al.* 1991). Cryopreservation methods were developed for meristems (Plessis *et al.* 1991, 1993, Wang *et al.* 2000, 2003, Matsumoto and Sakai 2000, 2003, Zhao *et al.* 2001, Benelli *et al.* 2003, Fabbri *et al.* 2008) or suspension cultures of embryogenic cells (Dussert *et al.*, 1991, Wang *et al.* 2002, 2004, Miaja *et al.* 2004, Gonzalez-Benito *et al.* 2008). Promising results were obtained recently by Markovic *et al.* (2013). However, despite great efforts the cryopreservation is still not very widespread.

The wider use of cryopreservation methods can be encouraged by thermal analysis involvement. Thermal analysis allows the detection of phase transitions in plant tissues and the assessment of individual steps of cryoprotocol prior to the cryopreservation. Individual steps of cryoprotocol improve their tolerance against dehydration and gradually dehydrate them, so that the remaining water content allows maintaining the viability of the explants but the proportion of freezable water is minimal.

The aim of this study was to determine how the gradual dehydration by cryoprotective solutions affects changes in thermal properties and viability of grapevine explants in three grapevine cultivars.

Material and Methods

Explants of three grapevine varieties ('Blussard modry', 'Portugal modry' and 'Kerner') were grown on a Quoirin-Lepoivre multiplication medium (Quoirin and Lepoivre 1977) with 34.2 $g \cdot L^{-1}$ sucrose, 6 $g \cdot L^{-1}$ agar and 0.2 $mg \cdot L^{-1}$ IAA pH 5.5-5.9 at 25 °C with a light intensity of 40 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and 16-h light period for 2 months. Subsequently, 2-3 nodal cuttings of at least 3 cm length were made and placed on the multiplication medium mentioned above and cultivated for next 10 days. The following vitrification procedure was based on the modification of the PVS3 solution (Nishizawa *et al.* 1993) and modified explant preculture with sucrose (Wang *et al.* 2003). Isolated shoot tips with size of 1-2 mm were hardened within three days on media with increasing concentrations of sucrose (0.3 M, 0.5 M and 0.75 M) in one-day intervals. Explants were successively dehydrated in thirty-minute intervals using a series of weak cryoprotective solutions: LS - Loading Solution (2 M glycerol, 0.4 M sucrose) (Sakai *et al.* 1991), LSV - *Vitis* Loading Solution (2 M glycerol, 0.75 M sucrose) (Wang *et al.* 2003) and PVS3(50) (25 % (w/v) glycerol, 25 % (w/v) sucrose; *i.e.* 50 % concentration of the original PVS3 (Nishizawa *et al.* 1993). Finally, the explants were exposed to strong cryoprotective solution PVS3(80) (40 % (w/v) glycerol, 40 % (w/v) sucrose; *i.e.* 80 % concentration of the original solution PVS3) for 60, 90 or 120 min. Regeneration of explants was determined after stepwise dehydration of explants on a regeneration medium of the same composition as the multiplication medium but supplemented with 0.25 $mg \cdot L^{-1}$ IBA instead of IAA. Shoot tips exposed to the strong cryoprotective solution PVS3(80) were unloaded by 0.75 M sucrose solution for 30 min before their transfer on the regeneration

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medium. The melting point temperature and the proportion of freezable water (percent of the fresh weight) were determined using a differential scanning calorimeter Q2000 with LNCS (TA Instruments, USA) in the temperature range from -140 to +20 °C at cooling and heating rates of 10 °C·min^{-1}, with the use of aluminium, hermetically sealed pans.

Statistically significant differences between varieties were evaluated by analysis of variance and Tukey's HSD (honest significant difference) test at a significance level $\alpha = 0.05$ using data analysis software system STATISTICA, version 12 (StatSoft, Inc., 2013). At least ten plants were used in each of three independent experiments. The relative frequencies were normalized using the arcsine transformation before ANOVA.

Results and Discussion

The explants were hardened during pre-culture by means of increasing concentrations of sucrose, dehydrated successively by weak cryoprotective solutions and finally dehydrated by a strong cryoprotective solution. Viability of grapevine explants was evaluated as a percentage of plants that were able to regenerate after each step of dehydration. The results indicated that regeneration of explants after stepwise dehydration was influenced by genotype (Fig. 1).

Analysis of variance showed statistically significantly lower regeneration ability of explants in variety 'Kerner' during dehydration as compared with other varieties. The decrease of regeneration ability of this variety occurred in the course of pre-culture of explants and continued until the end of the gradual dehydration (Fig. 1). The other varieties showed no significant changes in regeneration abilities, which ranged from 80 to 100 %. The most sensitive variety 'Kerner' responded to the application of cryopreservation procedure by reducing the regeneration ability up to 60 %, but this value does not exclude utilization of this variety in the cryoprotocol. Regeneration ability of other varieties was not a limiting factor for the application of the tested cryopreservation method.

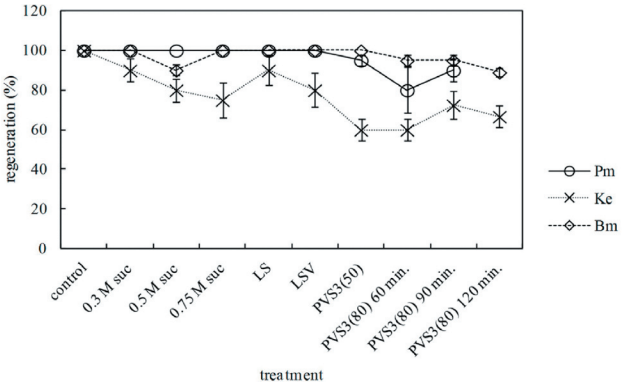

Fig. 1: Regeneration of grapevine explants after preculture on medium with increased sucrose content (0.3 M, 0.5 M, 0.75 M), after gradual dehydration by weak cryoprotectants (LS, LSV, PVS3(50)) and strong dehydration by the PVS3(80) for 60, 90 and 120 min (PVS3(80) 60 min, PVS3(80) 90 min, PVS3(80) 120 min) in three varieties: 'Portugal modry' (Pm), 'Kerner' (Ke) and 'Blussard modry' (Bm).

Dehydration of explants resulted in a decrease of melting point of shoot tips during their dehydration (Fig. 2). Melting point of explants decreased during dehydration in all varieties tested, but the decrease stopped after 60 min dehydration by the strong cryoprotectant PVS3(80). Analysis of variance showed that the melting point was statistically significantly lower in the variety 'Blussard modry' than in the other varieties due to the higher melting point depression after shoot tips treatment by the strong cryoprotective solution PVS3(80).

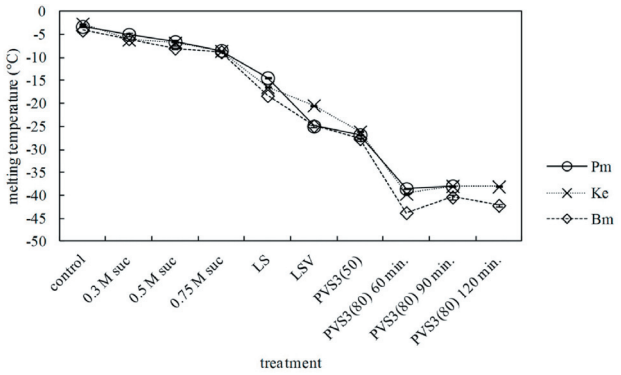

Fig. 2: Effect of pre-culture on media with an increased sucrose content (0.3 M, 0.5 M, 0.75 M), progressive dehydration by weak cryoprotectants (LS, LSV, PVS3(50)) and strong dehydration by PVS3(80) for 60, 90 and 120 min (PVS3(80) 60 min, PVS3(80) 90 min, PVS3(80) 120min.) on melting temperature in three varieties: 'Portugal modry' (Pm), 'Kerner' (Ke) and 'Blussard modry' (Bm).

The decrease of the melting temperature of explants during dehydration was connected with a decrease of the proportion of freezable water (Fig. 3). In this case, the decrease in this parameter was similar in all varieties but using analysis of variance proved a statistically ($p < 0.05$) higher portion of freezable water in variety 'Blussard modry' as compared with the other varieties. Most freezable water was removed during preculture and mild dehydration by weak cryopretective solutions but following decrease in the proportion of freezable water after 60 min of treatment with the PVS3(80) was very slow. This slow decrease allows terminating the dehydration of explants at the appropriate time without the risk of excessive dehydration of samples that would cause their damage.

Conclusion

The thermal analysis of plant material during vitrification procedure helps understanding the changes in plant material during successive dehydration. Slow, successive dehydration is a prerequisite for successful cryoprotocol application. The speed of plant dehydration and final level of freezable water in plant tissues can be evaluated by the thermal analysis. Slow water decrease during dehydration and its effect on explants thermal characteristics and vi-

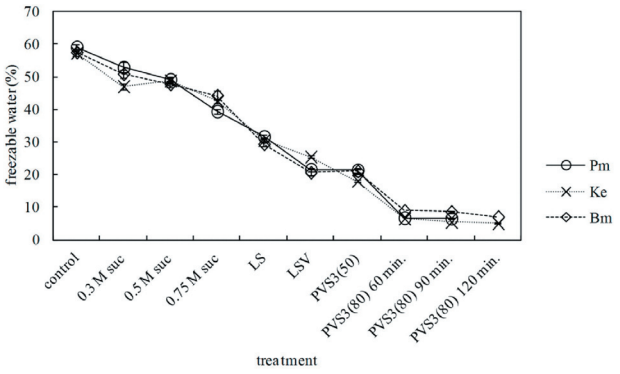

Fig. 3: Effect of pre-culture on a medium with an increased sucrose content (0.3 M, 0.5 M, 0.75 M), progressive dehydration by weak cryoprotectants (LS, LSV, PVS3(50)) and strong dehydration by PVS3(80) for 60, 90 and 120 min (PVS3(80) 60 min, PVS3(80) 90 min, PVS3(80) 120 min) on proportion of freezable water (percent of the fresh weight) in three varieties: 'Portugal modry' (Pm), 'Kerner' (Ke) and 'Blussard modry' (Bm).

ability was demonstrated in three grapevine varieties. The most sensitive to dehydration was variety 'Kerner' but its final regeneration ability still can allow cryopreservation of this genotype by the method tested. It could be concluded that the tested cryoprotocol could be used for cryopreservation of a range of varieties with different sensitivity to dehydration.

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