Plant Cell Reports (2000) 19:1064–1068

CELL BIOLOGY AND MORPHOGENESIS

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Cryopreservation of in vitro sugar beet (*Beta vulgaris* L.) shoot tips by a vitrification technique

Received: 13 September 1999 / Revision received: 2 March 2000 / Accepted:16 March 2000

Abstract Sugar beet shoot tips from cold-acclimated plants were successfully cryopreserved using a vitrification technique. Dissected shoot tips were precultured for 1 day at 5 °C on solidified DGJ0 medium with 0.3 M sucrose. After loading for 20 min with a mixture of 2 M glycerol and 0.4 M sucrose (20 °C), shoot tips were dehydrated with PVS2 (0 °C) for 20 min prior to immersion in liquid nitrogen. Both cold acclimation and loading enhanced the dehydration tolerance of shoot tips to PVS2. After thawing, shoot tips were deloaded for 15 min in liquid DGJ0 medium with 1.2 M sucrose (20 °C). The optimal exposure time to both loading solution and PVS2 depended on the in vitro morphology of the clone. With tetraploid clones a higher sucrose concentration during cold acclimation and preculture further enhanced survival after cryopreservation. Survival rates ranged between 60% and 100% depending on the clone. Since only 10–50% of the surviving shoot tips developed into non-hyperhydric shoots, regrowth was optimized.

Key words *Beta vulgaris* L. Cryopreservation · Shoot tips · Encapsulation-dehydration · Vitrification

Abbreviations *BA*: N-6-benzylaminopurine · *CMS*: Cytoplasmic male sterile · *DMSO*: Dimethylsulfoxide · *DGJ*: Medium of De Greef and Jacobs (1979) · *DGJ0*: Medium of De Greef and Jacobs (1979) lacking BA · *IBA*: Indole-3-butyric acid · *kin*: 6-Furfurylaminopurine · *LS*: Loading solution · *PAR*: Photosynthetic active radiation · *PVS2*: Plant vitrification solution · *RS*: Recovery solution

Communicated by P. Debergh

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Introduction

In most sugar beet (*Beta vulgaris* L.) breeding companies CMS lines, inbred O-type lines and tetraploid pollinators are stored as in vitro plants at a low temperature of 5 °C (Miedema 1982). However, the maintenance of these collections is laborious and time-consuming and, additionally, interesting genotypes can be lost due to contamination or human error (Towill 1988). To overcome some of these problems, attempts have been made to optimize cryopreservation techniques to store sugar beet germplasm. Root tips from hairy root cultures of transformed sugar beet were cryopreserved using a slow freezing method (Benson et al. 1991). Sugar beet shoot tips have been cryopreserved using both a slow freezing method (Braun 1988) and an encapsulation-dehydration technique (Vandenbussche and De Proft 1996). The best results were obtained with encapsulation-dehydration, after a three-step preculture and dehydration of the beads to a water content of approximately 20%. According to Vandenbussche and De Proft (1998) shoot tips are only sufficiently tolerant to dehydration if they are dissected from cold-acclimated in vitro shoots; survival rates after freezing ranged between 45% and 90% depending on the clone. One disadvantage of the optimized encapsulation-dehydration technique is that the cryopreservation procedure lasts 5 days and involves a 24-h pretreatment phase, a 3-day preculture and a 7-h dehydration period (Vandenbussche and De Proft 1996). With some sugar beet clones survival was less than 50%. Encapsulation retarded regrowth of shoot tips (Vandenbussche 1998). To address some of these drawbacks, we designed the study reported here to test a vitrification technique to cryopreserve sugar beet shoot tips. Meristems of numerous plant species have been

successfully cryopreserved using a vitrification procedure (Yamada et al. 1991; Towill and Jarret 1992; Matsumoto et al. 1995; Takagi et al. 1997).

Materials and methods

Plant material

In vitro control plants of 18 different sugar beet clones were grown on solidified DGJ-multiplication medium with 0.2 mg l^{-1} BA, 30 g l^{-1} sucrose and 6 g l^{-1} Difco Bacto agar (De Greef and Jacobs 1979). The pH was adjusted to 6.0 before autoclaving for 15 min at 121 °C. Plants were kept at a temperature of 19° ± 2 °C under a 16-h photoperiod with a light intensity of 60 μmol m^{-2} s^{-1} PAR supplied by white fluorescent lamps (Sylvania F36WT8/2084).

Some in vitro control plants of clone SES2 were grown on a modified DGJ medium supplemented with 0.2 mg l^{-1} kin, 30 g l^{-1} sucrose and 6 g l^{-1} Difco Bacto agar. The same culture conditions were applied as described above.

All of the clones tested were diploid genotypes except for SESB301, SESB89, SESC46, SESC54, SESF1, SES4S, SES5S and VDH 94, which were tetraploid.

In vitro plants were cold-acclimated by exposing them for 1 week to 5° ± 2 °C for 8 h a day at a light intensity of 60 μmol m^{-2} s^{-1} PAR (Vandenbussche and De Proft 1998). The DGJ medium used during cold acclimation was enriched with 0.3 M sucrose. Sugar beet shoot tips (1–2 mm) were dissected from both control and cold-acclimated plants and cryopreserved using an optimized encapsulation-dehydration technique (Vandenbussche and De Proft 1996) and a vitrification method.

For rooting, 4-week-old in vitro plants were placed on half-strength DGJ medium supplemented with 3 mg l^{-1} IBA, 30 g l^{-1} sucrose and 6 g l^{-1} Difco Bacto agar. After 5–6 weeks plants were transferred to soil and grown initially for 2 weeks under a mist tunnel. Plants were further grown under normal greenhouse conditions.

Encapsulation-dehydration technique

Shoot tips were pre-treated for 1 day on sterile filter paper (Schleicher & Schuell, 90-mm diam.) moistened with 2.5 ml of liquid DGJ0 medium supplemented with 30 g l^{-1} sucrose, in petri dishes (90-mm diam.) at 5° ± 2 °C. For encapsulation, shoot tips were suspended in liquid DGJ medium free of calcium and supplemented with 3% sodium alginate (Fluka, Biochemika). This mixture was dispensed with a 5-ml pipette by dropping it into liquid DGJ medium supplemented with 100 mM $CaCl_2$. After 30 min, beads (4-mm diam.) were stepwise precultured in liquid DGJ medium with 0.2 mg l^{-1} BA and different sucrose concentrations (0.3 M, 0.75 M and 1.0 M). A three-step preculture was applied, each step lasting 24 h. Beads were then placed on sterile filter paper (Schleicher & Schuell, 70-mm diam.) in a 200-ml glass jar filled with 50 g of dry silica gel. The jars were sealed well with a plastic lid and placed at a temperature of 19° ± 2 °C. Beads were dehydrated to a water content of 20–21% and then transferred to cryotubes (1.8 ml) prior to immersion in liquid nitrogen. After storage for at least 60 min, encapsulated shoot tips were thawed rapidly (1–2 min) in a water bath at 38 °C. Finally, the beads were placed in petri dishes (90-mm diam.) containing a solidified DGJ medium with 0.12 mg l^{-1} BA, 30 g l^{-1} sucrose and 6 g l^{-1} Difco Bacto agar (pH 6.0). The same culture conditions were applied as described earlier. Survival was evaluated by means of a stereomicroscope 3 weeks after cryopreservation. Survival percentages of the control and cryopreserved shoot tips reflected the number of shoot tips forming shoots relative to the total number of dehydrated or cryopreserved shoot tips (Vandenbussche and De Proft 1996).

Vitrification procedure

Dissected shoot tips were precultured for 1 day on a solidified DGJ0 medium with 0.3 M or 0.4 M sucrose and 6 g l^{-1} Difco Bacto agar. Meristems were wrapped in tissue paper (1.5 × 1.5 cm) and dehydrated with PVS2 either directly or after loading with 2 ml of a mixture of 2 M glycerol and 0.4 M sucrose (Matsumoto et al. 1994). Loading occurred in petri dishes (90-mm diam.) at 20 °C for various lengths of time (0–25 min), and wrapped shoot tips were blotted dry on sterile filter paper (Schleicher & Schuell, 70-mm diam.) before dehydration with PVS2. Wrapped shoot tips were then put into cryotubes (1.8 ml) filled with 1 ml of ice-cold PVS2. This vitrification solution consisted of 30% (w/v) glycerol, 15% (w/v) ethylene glycol and 15% (w/v) DMSO dissolved in a 0.4 M sucrose solution (Sakai et al. 1990). After 5–15 min, meristems were transferred to cryotubes (1.8 ml) containing 0.7 ml of ice-cold PVS2 for an additional 5–20 min of dehydration. The total dehydration time with PVS2 ranged between 10 min and 35 min. Cryotubes were subsequently plunged in liquid nitrogen and stored there for at least 60 min. For thawing, cryotubes were warmed rapidly (1–2 min) in a water bath at 38 °C. Wrapped shoot tips were transferred to petri dishes (90-mm diam.) and kept for 15 min at 20 °C in 2 ml of recovery solution. This solution consisted of liquid DGJ0 medium with 1.2 M sucrose (pH 6.0). Finally, meristems were cultured on two sterilized filter papers (Schleicher & Schuell, 70-mm diam.) placed on solidified DGJ0 medium with 0.3 M or 0.4 M sucrose and 6 g l^{-1} Difco Bacto agar (pH 6.0). After 2 days, the meristems were transferred to petri dishes (90-mm diam.) containing solidified DGJ medium with 0.12 mg l^{-1} BA, 30 g l^{-1} sucrose and 6 g l^{-1} Difco Bacto agar (pH 6.0). Culture conditions were similar to those previously described. Survival was evaluated as explained earlier. Regrowth of control and cryopreserved shoot tips was assessed as the number of regenerated non-hyperhydric shoots relative to the total number of dehydrated or cryopreserved shoot tips.

Statistical analysis

For the encapsulation-dehydration technique, 75 alginate-coated shoot tips were used in each experiment. Twenty beads were precultured and dehydrated as a control treatment, while 30 beads were used to test survival after cryopreservation. The remaining beads were used to determine the water content (Vandenbussche and De Proft 1998). When optimizing a vitrification technique, we used only 50 shoot tips in each experiment. Twenty shoot tips were loaded and/or dehydrated with PVS2 as a control treatment, while the remaining 30 shoot tips were cryopreserved. With both techniques, each experiment was repeated once.

The survival and regrowth percentages shown in the tables and figures are the means ± the standard derivation (SD) of two experiments. The data were analysed with a logistic regression model, allowing comparison of results with a χ^2-test. A significance level of P_r: $\chi^2 > 0.05$ was used.

Results and discussion

In general, cells will only survive low temperatures if the intracellular solutions vitrify during cooling. Consequently different techniques have been applied to induce a vitrification state in cells during cooling in order to cryopreserve plant material. With an encapsulation-dehydration technique intracellular solutions are concentrated by treatment with high molarity sugars, such as sucrose, followed by air drying (Fabre and Dereuddre 1990) or by dehydration with silica gel

(Niino and Sakai 1992). Cells can also be dehydrated with a concentrated solution (e.g. PVS2), which is the underlying mechanism of vitrification techniques (Uragami et al. 1990). The problem with both techniques, however, is the sensitivity of the plant material to dehydration. With cryopreservation of sugar beet shoot tips using an optimized encapsulation-dehydration technique, tolerance to dehydration enhanced by cold acclimation (Vandenbussche and De Proft 1998).

Cold-acclimated sugar beet shoot tips were sensitive to dehydration with PVS2 (Table 1). Since shoot tips of SES2 only survived cryopreservation after 20 and 25 min of dehydration, survival rates were low (P_r: 0.0162 and 0.013, respectively). For the results to improve, the tolerance of cold-acclimated shoot tips to dehydration with PVS2 had to be enhanced. A loading treatment prior to the 20 min of dehydration with PVS2 clearly increased the survival of both control and cryopreserved sugar beet shoot tips (P_r: 0.0001, Table 2). With shoot tips of *Wasabia japonica* (Matsumoto et al. 1994), *Lilium japonicum* (Matsumoto et al. 1995), *Colocasia esculenta* (Takagi et al. 1997) and *Ananas comosus* (González-Arnao et al. 1998), survival after cryopreservation also increased upon loading with a mixture of 2 M glycerol and 0.4 M sucrose. A 1-day preculture of the shoot tips before loading and 20 min of dehydration with PVS2 further increased survival after cryopreservation (Table 2, P_r: 0.0058). The dehydration tolerance of control shoot tips, however, was not influenced by preculture (Table 2). Pre-conditioning of shoot tips on a medium enriched with sucrose is beneficial in many tropical species in that it enhances the dehydration tolerance (Matsumoto et al. 1994; Takagi et al. 1997). The influence of pre-conditioning on the dehydration tolerance of cold-acclimated shoot tips, however, remains ambiguous (Matsumoto et al. 1995; Kuranuki and Sakai 1995).

The optimal dehydration time with PVS2 after preculture and 20 min of loading was subsequently determined (Fig. 1). The highest survival rates of cryopreserved shoot tips of SES2 were obtained after dehydration with PVS2 for 20–30 min (P_r: 0.0013).

Cold acclimation of in vitro sugar beet plants proved to be essential for successful cryopreservation with both an optimized encapsulation-dehydration (Vandenbussche and De Proft 1998) and the vitrification technique. Survival after cryopreservation of non-cold-acclimated vitrified shoot tips was significantly lower than that of cold-acclimated shoot tips (Table 2, P_r: 0.0001). Cold acclimation enhanced the dehydration tolerance, since only 72% ± 7% of the non-cold-acclimated control shoot tips survived (Table 2). The influence of cold acclimation on the dehydration and freezing tolerance of shoot tips can be partly explained by an accumulation of sugars and changes in the fatty acid composition of sugar beet shoots during cold acclimation (Vandenbussche et al. 1999). Proteins are also involved in increasing the dehydration and freezing tolerance of plants (Hughes and Dunn 1996).

The application of a vitrification technique often enhances survival rates after cryopreservation

Table 1 Effect of dehydration with PVS2 on survival of control and cryopreserved shoot tips from cold-acclimated plants of *B. vulgaris* L. (clone SES2). Shoot tips were deloaded for 15 min in RS. Fifty shoot tips were treated in each of two replicates

Treatment with PVS2 (min)	Survival rate (%) ± SD	
	Control	Cryopreserved
0	100	0
10	40 ± 8	0
20	38 ± 7	11 ± 4
25	33 ± 7	6 ± 3
30	5 ± 3	0

Table 2 Effect of cold acclimation (CA) (1 week 5 °C, 8-h day), preculture (1 day on DGJ0 with 0.3 M sucrose) and loading (mixture of 2 M glycerol and 0.4 M sucrose) on survival of control and cryopreserved shoot tips of *B. vulgaris* L (clone SES2). Shoot tips were dehydrated for 20 min with PVS2 and deloaded for 15 min in RS. Fifty shoot tips were treated in each of two replicates

CA	Preculture (day)	Loading treatment (min)	Survival rate (%) ± SD	
			Control	Cryopreserved
+	0	20	92 ± 4	72 ± 6
+	1	10	100	92 ± 4
+	1	20	100	90 ± 4
−	1	20	72 ± 7	43 ± 6

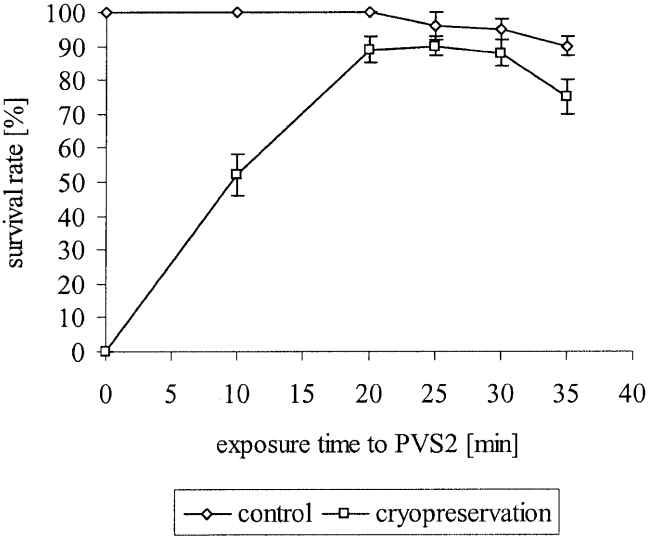

Fig. 1 Optimization of the dehydration time with PVS2 of control and cryopreserved shoot tips of *B. vulgaris* L. (clone SES2) after preculture (1 day on DGJ0 with 0.3 M sucrose) and a 20-min loading treatment (mixture of 2 M glycerol and 0.4 M sucrose). Shoot tips were deloaded for 15 min in RS. Fifty shoot tips were treated in each of two replicates. *Bars:* Standard deviation

compared to other methods (Kuranuki and Sakai 1995; González-Arnao et al. 1998). The opposite has been reported with shoot tips of *Prunus dulcis* M (Shatnawi et al. 1999). With sugar beet shoot tips the use of a vitrification technique further increased survival of clone SES2 (Table 3) but did not alter the survival rates of clone SES1.

Shoot tips of 16 other sugar beet clones were also cryopreserved with the optimized vitrification technique (Table 3). With shoot tips of clones SESB2 and SESB301, results were improved by prolonging the dehydration period with both the loading solution and PVS2 (P_r: 0.0033 and 0.0024, respectively). Both clones had thicker leaves and petioles, which resulted in larger sized apices (visual observation). When an encapsulation-dehydration technique was used, survival of shoot tips from a clone of a similar morphology was improved by increasing the sucrose concentration in the medium during cold acclimation (Vandenbussche 1998). With a vitrification technique, the influence of a higher sucrose concentration (0.4 M) during cold acclimation and preculture was less distinct. Only with shoot tips of the tetraploid clone SESB301 did survival after cryopreservation increase (P_r: 0.0001). Although the diploid clone SESB2 had a similar morphology, survival after cryopreservation was not altered (Table 3). This modified vitrification method, using 0.4 M sucrose during cold acclimation and preculture, was also successfully applied to 7 other tetraploid clones (Table 3).

Whatever the cryopreservation technique used, sugar beet shoot tips lost their green colour within 1 day after thawing. After 2–3 days, surviving shoot tips regained their green colour, and new green leaves were visible after 7 days of regrowth. Only direct shoot formation without intermediate callus formation was observed. After vitrification, subsequent regrowth of the shoot tips proceeded faster than with encapsulation-dehydration. After 6–8 weeks, regenerated plants had grown sufficiently (height of 1.0 cm) to be transferred to solidified DGJ-multiplication medium. With an encapsulation-dehydration technique this developmental stage was only reached after 10–14 weeks. After cryopreservation with a vitrification method, only 32% ± 8% of the shoot tips of clone SES2 developed into non-hyperhydric shoots. With encapsulation-dehydration, 56% ± 6% of the cryopreserved shoot tips of clone SES2 had a non-hyperhydric morphology. Hyperhydric sugar beet shoots had thicker, curled leaves with a gritty surface. Shoots were pale and apex necrosis was often observed. When different clones were compared between 10% and 50% of the shoot tips developed into non-hyperhydric shoots after cryopreservation with the vitrification method. However, results were improved by lowering the BA concentration in the medium during regrowth of both control and cryopreserved shoot tips. After regrowth on solidified DGJ medium with only 0.1 mg l^{-1} BA, 46% ± 7% of the cryopreserved shoot tips of SES2 developed into non-hyperhydric shoots (P_r: 0.0091). Regrowth was further improved by growing in vitro plants of SES2 for 4 weeks on a modified DGJ medium with 0.2 mg l^{-1} kin prior to cold acclimation. Plants were cold acclimated as previously described, but BA was omitted from the medium. As many as 97% ± 2% of the shoot tips dissected from these plants survived cryopreservation with the optimized vitrification method, and 73% ± 6% developed into non-hyperhydric shoots (P_r: 0.0001). These modifications remain to be tested with other clones. Control and cryopreserved regenerated shoots of clones SES2 and SEH303 were rooted, acclimated and grown in the greenhouse. No phenotypic changes were observed in the recovered plants.

In conclusion, the optimized vitrification technique is recommended as the means to cryopreserve sugar beet shoot tips since it proved to be easier and faster. Only 2 days were needed to cryopreserve sugar beet shoot tips compared to 5 days with an optimized encap-

Table 3 Survival rates of control and cryopreserved shoot tips of different clones of *Beta vulgaris* L. An encapsulation-dehydration (Encap-dehydr) technique and a vitrification method were used. During cold acclimation (CA) in vitro plants were held at 5 °C for 1 week (8-h day). Shoot tips were precultured at 5 °C for 1 day. Fifty shoot tips were treated in each of two replicates

Clone	Sucrose concentration during CA and preculture (M)	Cryogenic protocol	Survival rate (%) ± SD	
			Control	Cryopreserved
SES2	0.3	Encaps-dehydr[a]	65 ± 10	60 ± 9
SES2	0.3	Vitrification[b]	100	90 ± 4
SES1	0.3	Encaps-dehydr[a]	74 ± 9	69 ± 6
SES1	0.3	Vitrification[b]	95 ± 3	74 ± 6
SESC6b	0.3	Vitrification[b]	97 ± 2	80 ± 5
SESB34	0.3	Vitrification[b]	95 ± 5	74 ± 8
SESD20	0.3	Vitrification[b]	100	85 ± 7
SESH195	0.3	Vitrification[b]	100	83 ± 7
SEH303	0.3	Vitrification[b]	82 ± 8	81 ± 7
SES414	0.3	Vitrification[b]	95 ± 5	80 ± 7
SESB2	0.3	Vitrification[b]	44 ± 12	41 ± 9
SESB2	0.3	Vitrification[c]	89 ± 7	63 ± 9
SESB2	0.4	Vitrification[c]	95 ± 5	67 ± 9
SESB301	0.3	Vitrification[b]	83 ± 6	28 ± 6
SESB301	0.3	Vitrification[c]	78 ± 9	60 ± 9
SESB301	0.4	Vitrification[c]	100	83 ± 5
SESB4	0.4	Vitrification[c]	95 ± 5	80 ± 7
SESB89	0.4	Vitrification[c]	100	63 ± 9
SESC46	0.4	Vitrification[c]	95 ± 5	73 ± 8
SESC54	0.4	Vitrification[c]	94 ± 6	61 ± 9
SESF1	0.4	Vitrification[c]	90 ± 6	68 ± 8
SES4S	0.4	Vitrification[c]	100	90 ± 5
SES5S	0.4	Vitrification[c]	100	72 ± 8
VDH94	0.4	Vitrification[c]	100	80 ± 7

[a] Encapsulated shoot tips were stepwise precultured in liquid DGJ medium supplemented with 0.3 M, 0. 75 M and 1.0 M sucrose, and the beads were dried for 7 h on silica gel prior to immersion in liquid nitrogen

[b] Shoot tips were loaded for 20 min at 25 °C and dehydrated with PVS2 for 20 min at 0 °C before immersion in liquid nitrogen

[c] Shoot tips were loaded for 25 min at 25 °C and dehydrated with PVS2 for 30 min at 0 °C before immersion in liquid nitrogen

sulation-dehydration technique. Furthermore when different clones were compared, survival after cryopreservation ranged between 60% and 100%. With shoot tips of clone SES2, higher survival rates were obtained with the vitrification method than with encapsulation-dehydration. Finally, regrowth was faster when the optimized vitrification technique was applied.

Acknowledgements The authors thank ADVANTA for providing facilities and supplying the sugar beet clones. The advice on the statistical analysis by Prof. J Beirlant was greatly appreciated. This research was financed by a grant of the Flemish Institute to promote Scientific/Technological Research in the Industry, Brussels.

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