International Journal of Tropical Agriculture
Volume 40, Numbers (3-4) • 2022 : pp. 355-361
ISSN: 0254-8755

Comparison of Three Cryoprotectants with V-Cryoplate Droplet Vitrification Technique for Cryopreservation of Shoot Tips of *Stevia rebaudiana* Bertoni

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Abstract: This study directly compared the effects of three cryoprotectants with the V-Cryoplate Droplet Vitrification method for effective cryobanking of *Stevia rebaudiana*, a medicinally important plant. Shoot cuttings of 5-6 cm were collected from the mother plant at the University of Mississippi and surface disinfected in 15% commercial bleach with 0.1% Tween 20 for 10 min, rinsed three times with sterile distilled water and also treated with 0.2% mercuric chloride for 3 min. The shoot cuttings were again rinsed three times with sterile distilled water before planting in a multiplication medium. After 4 weeks, shoot tips (~0.5 mm) with one or two leaf primordia were aseptically dissected and pretreated for 48 hours in MS-agar medium with 0.3 M sucrose and 5% DMSO followed by loading of cells with 2 M glycerol in 0.4 M sucrose MS medium. Cryoprotectants (plant vitrification solutions (PVS) #2, #3 and #4) were initially screened at 25°C for 10-30 min. Subsequent experiments were based on 15 min exposure duration. Regrowth after a 15 min exposure to PVS2 prior to liquid nitrogen (60%) or PVS4 (61%) were significantly higher than those of other treatments. Regrowth of cryopreserved shoot tips was significantly higher with PVS4 (64%) compared to PVS2 (54%) and PVS3 (3%). These results suggest that the use of PVS4 as a cryoprotectant in the V-Cryoplate Droplet Vitrification technique was most effective for cryopreservation of *Stevia* shoot tips. Furthermore, plants reproduced from cryopreserved shoot tips were found to be highly comparable to that of the mother plant in terms of chemical profile.

Keywords: Conservation, plant tissue culture, preculture, pretreatment, regrowth

INTRODUCTION

Stevia rebaudiana is a herbaceous perennial herb (2n=22) belonging to the Asteraceae family (Yadav *et al.* 2011). It is popularly used as natural high-potency sweetener in foods and drugs due to the presence of the active chemical components, stevioside and rebaudiosides (Yadav *et al.* 2011). *Stevia* plants are usually derived from seeds with low percentage germination and considerable yield variation (Shaffert and Chebotar, 1994). Several plant tissue culture protocols have been developed for improved *in-vitro* plant growth (Sivaram and Mukundan, 2003; Ahmed *et al.* 2007; Lata *et al.* 2013; Namdari *et al.* 2015). Tissue cultured *S. rebaudiana* had a high rate (95%) of *ex vitro* acclimatization (Chandra *et al.* 2012), and similar phytochemical profile to the mother plants (Lata *et al.* 2012). Zayova *et al.* (2017) reported an *in vitro* conservation protocol for *Stevia* using an osmoticum (mannitol) to slow growth which allowed storage of *in-vitro* germplasm for up to six months. Other temporary storage methods involve encapsulating *Stevia* nodal segments, shoot tips or buds with a synthetic coating (4-

Article History: Received: 05 August 2022; Revised: 19 August 2022; Accepted: 14 September 2022

5% sodium alginate/ 50-75 mM calcium matrix) for protective purposes before *in vitro* culture (Verma *et al.* 2012; Lata *et al.* 2013; Lata *et al.* 2014). The synthetic coating technique allowed *in-vitro* storage for up to 8 months (Lata *et al.* 2014a; Lata *et al.* 2014b).

Cryopreservation [storage of explants in liquid nitrogen (LN) at -196°C] provides much longer period of *in vitro* storage under safe and stable conditions (Reed 2008). Different protocols were reported involving plant vitrification solutions; PVS1 (Urugami *et al.*, 1989), PVS2 (Sakai *et al.*, 1990; Sakai 2000), PVS3, PVS4 and PVS5 (Nishizawa *et al.* 1993; Sakai 2000). Of these vitrification solutions, the PVS2, PVS3 and PVS4 are widely used for plant cryopreservation (Sakai and Engelman, 2007). Cryopreservation protocols reported for *S. rebaudiana* shoot tips include a two-step (80% and 100%) PVS2 treatment, followed by vitrification in LN which resulted in 66% regrowth (Shatnawi *et al.* 2011a). Shatnawi *et al.* (2011b) obtained up to 69% regrowth following pretreatment in 0.4M sorbitol for 48 hours prior to the two-step (80% and 100%) PVS2 pretreatment.

Modifications of the standard PVS2 vitrification method which, resulted in significant increases in regrowth, were reported for a number of medicinal plants. For example, Yamamoto *et al.* (2012) reported 73-100% regrowth of cryopreserved shoot tips of mint using an aluminum V-Cryoplate vitrification method which involves encapsulation of shoot tips in alginate gel on the aluminum V-Cryoplate prior to the application of PVS2 for 20 min at 25°C. Tanaka *et al.* (2014) obtained 80% regrowth of cryopreserved buds of *Chrysanthemum* following the V-Cryoplate vitrification method with PVS2 application for 40 min at 25°C.

This study aims to directly compare the effects of three cryoprotectants PVS2, PVS3, and PVS4 with a modified V-Cryoplate droplet vitrification technique to determine their specific effects or reliability for the long-term storage of *Stevia* shoot tips in LN at any germplasm bank.

MATERIALS AND METHODS

Source of Explant

The shoot cuttings (5-6 cm) of *Stevia rebaudiana* were obtained from the mother plant grown at the National Center for Natural Products Research (NCNPR), Coy Waller Laboratory Complex, University of Mississippi, USA.

Sterilization Method

The shoots were cut into segments of 2-3 cm. They were pre-rinsed three times in running tap water, followed by surface sterilization in 15% commercial bleach [Clorox, regular bleach (5.25% v/v)] with 0.1% Tween 20 for 10 min and then rinsed three times with sterile distilled water (500 ml). The shoot segments were also treated with 0.2% mercuric chloride (Sigma-Aldrich Co., St Louis, MO) for 3 min. Shoot segments were rinsed three times with sterile distilled water before planting in multiplication medium.

Composition of Multiplication Medium

The *in vitro* multiplication medium contained 4.33 gL^{-1} MS (Murashige and Skoog, 1962) mineral salts with Gamborg's vitamin formulated as a commercial powder (Caisson Laboratories Inc. Smithfield, UT, product #MSP01-100LT, LOT 10160003), MS vitamins solution (Caisson Laboratories Inc. product #MVL01-100ML), 0.12 mgL^{-1} N^6-(3-hydroxybenzyl) adenine (meta-Topolin, Caisson Laboratories Inc. product # T039-100MG), 30 gL^{-1} anhydrous sucrose (Caisson Laboratories Inc. product #S011-10KG), 8 gL^{-1} agar powder (Caisson Laboratories Inc. product #A038-1KG), and 1 gL^{-1} activated charcoal (Sigma-Aldrich Co., St Louis, MO. Product #C9157). The pH of this medium was adjusted to 5.7 before the addition of the agar and activated charcoal. Growth medium was sterilized in an autoclave at 121 °C (249 °F), 15 psi for 15 min. After sterilization, meta-Topolin was added to the cooled medium before medium got dispensed into Magenta GA7 boxes (Magenta Corp., Chicago, IL). The shoot segments were multiplied on fresh growth medium every 4 weeks.

Growth Chamber Conditions

All cultures were grown in a growth chamber (Percival Scientific Inc. IA, model E36LX) at 25 ± 1°C, 16 h photoperiod and photon flux of 52±2 µmol m^{-2} s^{-}1 (LI-250A, LI-COR® Biosciences, USA) provided by cool white fluorescent bulbs (Philips,

USA) before the shoot tips were aseptically removed for cryopreservation experiments.

Pretreatment Procedure

Pretreatment medium consisted of MS salts and vitamins, 0.3 M sucrose, 8 gL^{-1} agar and 5% dimethyl sulfoxide (DMSO, Sigma-Aldrich Co. CAS #67-68-5) at pH 5.7. The medium was sterilized as above. The DMSO was filter sterilized using membrane filters (0.45 µm, 150-ml analytical filter unit, Corning Life Sciences Inc., Pittsburgh, PA) and added to pretreatment medium after autoclaving. About 20 ml of medium was dispensed into each Petri dish (100 mm diameter x 15 mm height). Shoot tips (~0.5 mm) with meristematic dome surrounded by one or two attached leaf primodia were aseptically excised from 4-wk old shoot cultures and planted on the pretreatment plates for 48 hours in the growth chamber under the conditions described above.

Application of Loading Solution and Cryoprotectants

Pretreated shoot tips were transferred to 1 ml loading solution (LS) [2 M glycerol in 0.4 M sucrose MS medium (v/v), pH 5.8] in 1.2 ml cryovials (Nalgene® Thermo Fisher Scientific, Rochester, NY) for 20 min at 25 ± 1 °C (Sakai, 2000). The LS was removed and the shoot tips were individually transferred to 5 µl droplets of cryoprotectant on sterile aluminum V-Cryoplates (7mm x 37mm x 0.5 mm, indent diameter 1.5 mm, depth 0.75 mm) with 12 wells (Yamamoto *et al.*, 2012; Matsumoto, 2017).

Three cryoprotectants; PVS2 [3.26M glycerol, 15% ethylene glycol, 15% DMSO in liquid MS medium with 0.4 M sucrose (w/v),] (Sakai *et al.* 1990), PVS3 [1.46M sucrose, 5.43M glycerol in liquid MS medium (w/v),] (Nishizawa *et al.* 1993) and PVS4 [0.6 M sucrose, 3.8 M glycerol and 20% ethylene glycol in liquid MS (w/v)] (Sakai, 2000) were tested in two sets of experiments; 1) to determine the optimal exposure duration of explants to each cryoprotectant during cryopreservation and 2) to determine the most effective cryoprotectant for cryopreservation of *Stevia* using modified V-Cryoplate Vitrification. All three cryoprotectant solutions were adjusted to pH 5.8.

The shoot tips were treated with each cryoprotectant at 25 °C for 10, 15, 20, 25 and 30 min prior to immersion in LN for 10-15 min followed by rapid rewarming in 45°C water bath (Model 2829, Thermo Fisher Scientific, Marietta, OH) for 1 min. All samples were rinsed by serial dilution with liquid MS medium containing 1.2 M sucrose and then cultured in the multiplication medium described earlier. Shoot tips were planted on 2 ml of multiplication medium per well of a 24-cell plates (Costar, Cambridge, MA).

The V-Cryoplate droplet vitrification procedure for stevia

A modified V-Cryoplate droplet vitrification protocol without alginate gel was followed (Yamamoto *et al.* 2011; Yamamoto *et al.* 2012; Matsumoto, 2017). Shoot tips were; (a) planted in pretreatment medium for 48 hours under the conditions described above, (b) Shoot tips were treated in LS for 15 min, (c) the shoot tips were individually transferred into 5 µl droplets of each cryoprotectant (PVS #2, #3, and #4) on sterile aluminum V-Cryoplate. Each shoot tip was held in its cryoprotectant for 15 min, (d) each aluminum V-Cryoplate with shoot tips was transferred to a cryovial (without LN in it) and lid closed, (e) cryovials were rapidly plunged in LN for 10-15 mins, followed by rapid rewarming in 45 °C water for 1 min, (f) shoot tips were rinsed by serial dilution in liquid MS solution containing 1.2M sucrose, (g) control shoot tips had all treatments except LN and rewarming. Each cryoprotectant was tested separately, (h) all shoot tips were planted in the multiplication medium described above. This experiment was done with 20 shoot tips per treatment and three replicates (n=60). Controls had all treatments except LN exposure (12 per treatment with three replicates) (n=36).

Chemical Profiling, Rebaudioside A and Stevioside Content

Leaves of mother plant and plants reproduced from cryopreserved shoot tips were analyzed for Rebaudioside A and Stevioside content following the method described by Lata *et al.* 2013.

Data and Statistical Analysis

Data on percentage survival and regrowth of shoot tips were recorded after six weeks of cryopreservation. Shoot tips were considered as survived if each original shoot tip remained green and enlarged after cryopreservation. Shoot tips were considered as regrowth if in addition, the cryopreserved shoot tip resumed growth with production of leaves and shoots. Data were analyzed using ANOVA (SAS version 9.2 for windows; SAS Institute Inc., Cary, NC, USA). Means separation was done using Duncan's multiple range test. Differences in means were considered significant at 5% level of probability. The data were arcsine transformed before analysis.

RESULTS AND DISCUSSION

Effects of cryoprotectant duration on regrowth of Stevia shoot tips

There was a significantly higher regrowth of cryopreserved shoot tips exposed to PVS2 for 15 min (60%) compared to regrowth (≤50%) obtained following exposure of explants to other durations in PVS2 (Figure 1A). A similar regrowth response after cryopreservation was reported by Shatnawi *et al.* (2011a) who obtained 66% regrowth after exposure of *Stevia* shoot tips to PVS2 for 60 min. On the contrary, we observed zero regrowth beyond 20 min in PVS2. Differences between these results may be related to the fact that the authors applied PVS2 at 0°C while in the current study; it was applied in droplets at 25 °C. Temperature conditions influence uptake and penetration of PVS2 (Volk and Walters, 2006).

The cryopreserved shoot tips in PVS4 treatment showed significantly higher percentage regrowth after 15 min (61%) and 20 min (60%) treatments compared to other duration in PVS4 (Figure 1B). For subsequent experiments, PVS4 was applied for 15 min. Cryopreserved shoot tips treated with PVS3 had much lower regrowth (≤20%) (data not shown). The key to a successful cryopreservation with vitrification solutions is to carefully control chemical toxicity resulting from excessive osmotic stress. Therefore optimizing the exposure duration for each solution is critical to obtaining a high survival or regrowth following cryopreservation. This study did not test *Stevia* on PVS3 beyond 30 min however, Kim *et al.*, (2006) tested PVS3 with droplet vitrification on garlic explants using 90-150 min on PVS3 and determined that optimal regrowth occurred at 150 min. Also, performing this operation at 0°C may reduce or eliminate any injurious effects as shown by Thinh (1997).

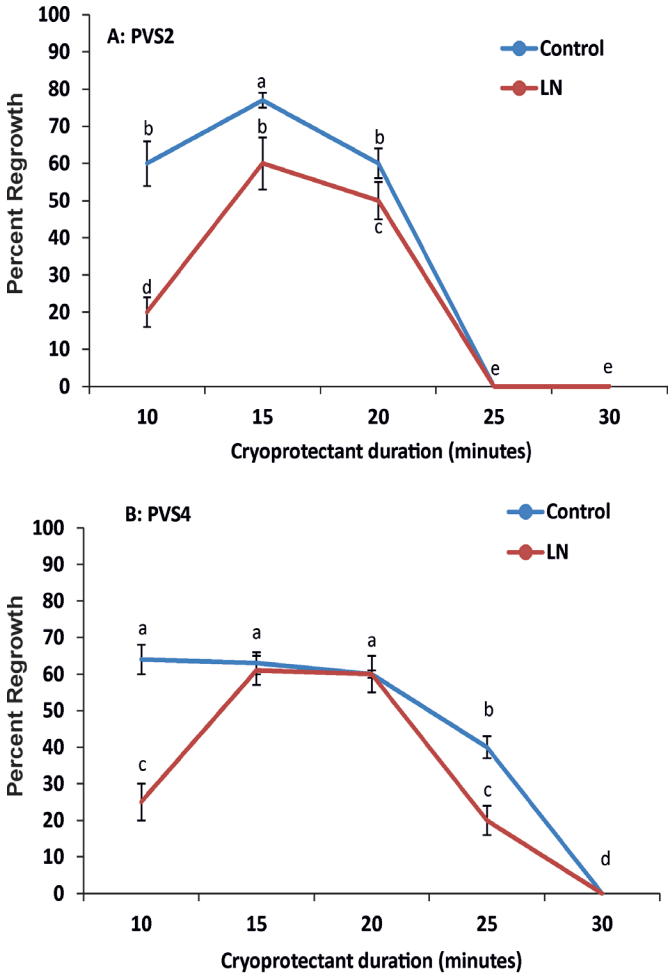

Figure 1: Regrowth response of shoot tips of *Stevia rebaudiana* exposed to (*A*) plant vitrification solution #2 (PVS2) (*B*) plant vitrification solution #4 (PVS4) for 10-30 min at 25±1 °C prior to cryopreservation in liquid nitrogen (LN). The controls had all treatments except LN. Regrowth data is defined as percentage of total number of original shoot tips that resumed normal growth into a plant after cryopreservation. Error bars represent the standard error of means. Means with the same letter are not significantly different at 5% level of probability

Effect of cryoprotectants on the survival and regrowth of Stevia shoot tips

The cryopreservation of *Stevia rebaudiana* shoot tips in LN using PVS4 with the V-Cryoplate droplet vitrification method resulted in 86% survival. This was significantly ($p < 0.001$) higher than survival of shoot tips treated with PVS2 (68%) and PVS3 (19%). The mechanisms by which cryoprotectants are effective towards the cryoprotection of shoot tips have been reported. Briefly, they displace cellular water, change the freezing behavior of any leftover intracellular water, prevent excessive water loss during dehydration etc. (Volk and Walters, 2006). Expectedly, there was a high survival rate (>80%) among the control shoot tips but no significant difference among these survivals (Figure 2A). Generally, a high percentage survival increases the chances of future plant recovery with hormone manipulation, than dead or damaged shoot tips.

The regrowth of vitrified shoot tips treated with PVS4 (64%) was significantly higher than PVS2 (54%) and PVS3 (3%). The regrowth of control shoot tips treated with PVS2 however was significantly higher (86%) than PVS4 (67%) and PVS3 (42%) (Figure 2B). Regrowth following cryopreservation of *Stevia* shoot tips was PVS4 >PVS2>PVS3. The PVS4 composition had higher glycerol, ethylene glycol and sucrose than PVS2 thus, appears super-viscous and more likely to vitrify upon contact with LN, sooner than PVS2. These vitrification agents structure intracellular water such that they are unlikely to freeze during cryopreservation (Wolfe *et al.* 2002). Yamamoto *et al.* (2011) reported 65-90% regrowth of cryopreserved shoot tips of *Dalmatian chrysanthemum* using composition of a cryoprotectant similar to PVS4 after embedding the shoot tips in alginate gel. Both the survival and regrowth percentages following cryopreservation of *Stevia* shoot tips by modified V-Cryoplate vitrification with PVS2 and PVS4 were both above 50% which far exceeds the 40% minimum recommended for storage of plant genetic resources (Reed, 2001; Reed *et al.* 2005). The original V-Cryoplate vitrification method with PVS2 produced 73-100% regrowth of cryopreserved shoot tips of mint (Yamamoto *et al.* 2012).

Regrowth of cryopreserved *Stevia* shoot tips with PVS3 was less than 5% (Figure 2B). In a similar comparative study involving the use of PVS2, PVS3 and PVS4 with droplet vitrification technique, Bustam *et al.* (2016) determined that no regrowth occurred with *C. latifolia* primary protocorms cryopreserved with PVS3. However, Zhumagulova *et al.* (2014) reported that PVS3 significantly improved the recovery or viability of cryopreserved dormant buds of pear compared to PVS2 or PVS4.

Chemical Profiling

Plants were reproduced after cryopreservation (by using PVS4 as a cryoprotectant in the V-Cryoplate Droplet Vitrification Technique)

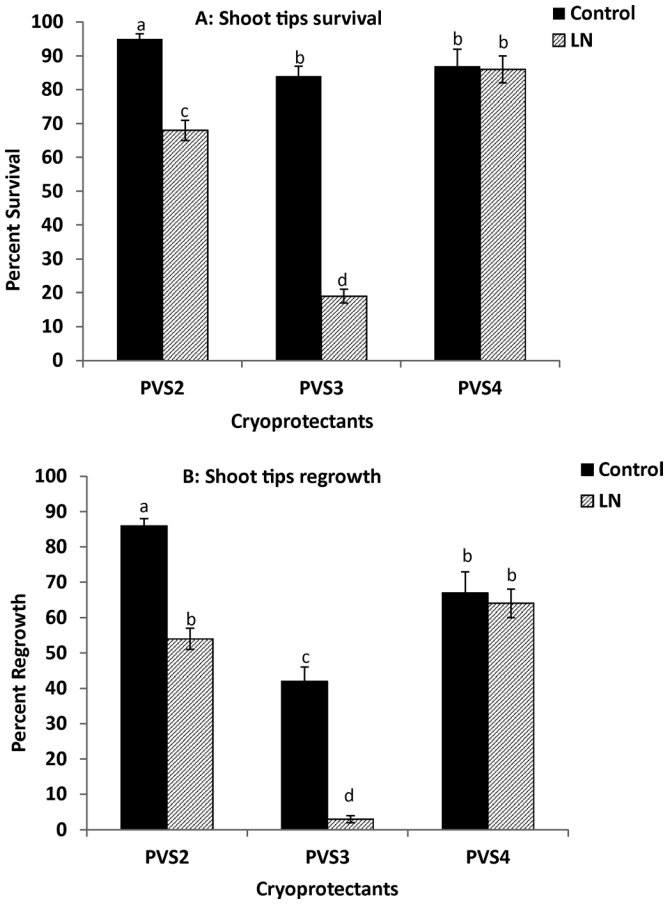

Figure 2: Direct comparison of the effects of three cryoprotectants (plant vitrification solutions; PVS2, PVS3 and PVS4) exposure for 15 min on the (A) survival and (B) regrowth of shoot tips of *Stevia rebaudiana* following cryopreservation in liquid nitrogen (LN) by a modified V-Cryoplate Droplet Vitrification. The controls had all treatments except liquid nitrogen. Means with the same letter are not significantly different at 5% level of probability.

Figure 3: Important stages of cryopreservation of *Stevia rebaudiana*: (a) aseptically, excised shoot tips (~0.5 mm) in pretreatment medium for 48 hours prior to cryopreservation, (b) regrowth of shoot tips six weeks after cryopreservation in liquid nitrogen, (c) three-week old recovered- plants growing in the multiplication medium and (d) eight-week old acclimatized plants derived from cryopreserved shoot tips.

and grown up to maturity. The biomass samples taken from cryopreserved and reproduced plants (n = 9) were compared with the samples of mother plant (n = 3) for the chemical profile. Plants reproduced from cryopreserved shoot tips (Rebaudioside A, 3.49 ± 1.08 and Stevioside 4.50 ± 0.99) were found to be highly comparable to that of the mother plant (Rebaudioside A, 3.18 ± 1.23; Stevioside 4.17 ± 1.29) (Figure 4). All acclimatized plants appear morphologically healthy with good vigor (Figure 3D).

To the best of our knowledge, this is the first study adapting this modified aluminum V-Cryoplate droplet vitrificaton cryopreservation technique to *Stevia rebaudiana* with specific information on how cryoprotectants compare in a modified V-Cryoplate droplet vitrification protocol following rewarming in 45°C water.

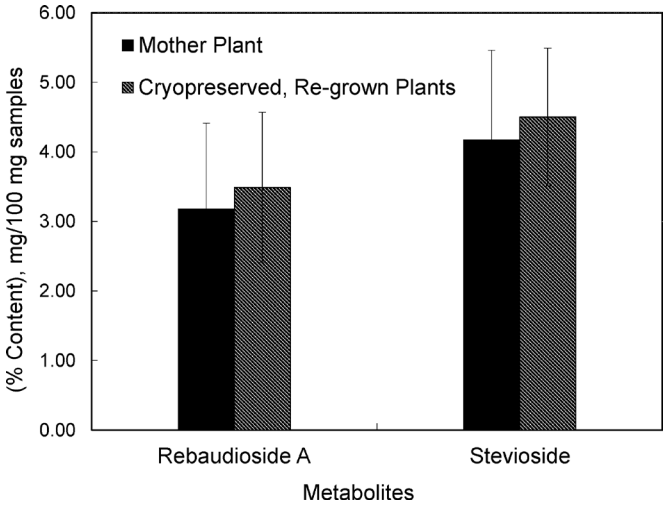

Figure 4: Comparison of Rebaudioside A and Stevioside content in mother plant and plants reproduced from cryopreserved shoot tips of *Stevia rebaudiana*

It took about 4 months from the initial explant culture to obtain acclimatized plants (Figure 3A-D).

Acknowledgements

The work was supported in part by United States Department of Agriculture, Agricultural Research Service (USDA-ARS), Specific Cooperative Agreement No. 58-606-6-015.

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