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Clonal propagation and cryogenic storage of the medicinal plant *Stevia rebaudiana*

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Abstract

Successful clonal propagation of *Stevia rebaudiana* was achieved using microshoots as a primary step for *in vitro* conservation. Maximum proliferation was obtained on Murashige and Skoog (MS) medium supplemented with 1.5 mg L^{-1} benzyl amino purine and 0.2 mg L^{-1} indole-3-butyric-acid (IBA). Auxin increased rooting percentage of shoots at concentration of 0.4 mg L^{-1} IBA, indole-3-acetic-acid or naphthalene acetic acid and no rooting occurred without plant growth regulator. A survival of 90% was achieved when rooted explants were acclimatized *in vivo* in 1 soil: 1 perlite: 1 peat. *In vitro S. rebaudian*a shoots were successfully stored for up to 32 weeks on MS medium supplemented with an appropriate concentration of sucrose, sorbitol or mannitol, at 24 ± 2°C. After 32 weeks, 93.6% of the shoots were able to survive. Moreover, 89.3% of them were able to regrow when stored under light conditions. Cryopreservation by vitrification was successfully achieved (65.6% regrowth) when shoot tips were precultured on a medium supplemented with 0.4 M sorbitol for 2 d, followed by loading shoot tips with 80% concentrated plant vitrification solution 2 (PVS2) for 20 min; then dehydrated with 100% PVS2 for 60 min at 0°C prior to storage in liquid nitrogen. This procedure is easy to handle and produced a high levels of shoot formation. This protocol could be useful for long-term storage of *S. rebaudiana* germplasm.

Additional key words: cryopreservation; *in vitro* conservation; micropropagation; osmoticum; root formation.

Resumen

Propagación clonal y almacenamiento criogénico de la planta medicinal *Stevia rebaudiana*

Se logró con éxito la propagación clonal de *Stevia rebaudiana,* utilizando microtallos como primer paso para su conservación *in vitro.* Se obtuvo la proliferación máxima en el medio Murashige y Skoog (MS) suplementado con 1,5 mg L^{-1} de bencil amino purina y 0,2 mg L^{-1} de ácido indol-3-butírico (IBA). Las auxinas aumentaron el porcentaje de enraizamiento de los brotes en la concentración de 0,4 mg L^{-1} de IBA, ácido indol-3-acético o el ácido naftalen acético, pero no se produjo enraizamiento sin regulador del crecimiento vegetal. Se logró una supervivencia del 90% cuando los explantes enraizados fueron aclimatizados *in vivo* en 1 suelo: 1 perlita: 1 turba. Se almacenaron con éxito brotes de *S. rebaudiana in vitro* hasta 32 semanas a 24 ± 2°C, en medio MS suplementado con una concentración apropiada de sacarosa, sorbitol o manitol. Después de 32 semanas, el 93,6% de los brotes fueron capaces de sobrevivir y el 89,3% fueron capaces de rebrotar cuando se almacenaron bajo condiciones de luz. Se logró con éxito (65,6% rebrotes) la criopreservación por vitrificación cuando los ápices se pre-cultivaron durante 2 días en un medio suplementado con sorbitol 0,4 M, y a continuación con una solución de vitrificación de la planta 2 (PVS2) al 80% durante 20 minutos, seguido de una deshidratación con PVS2 al 100% durante 60 min a 0°C antes de su almacenamiento en nitrógeno líquido. Este procedimiento es fácil de ejecutar y puede ser útil para el almacenamiento a largo plazo del germoplasma de *S. rebaudiana.*

Palabras clave adicionales: conservación *in vitro*; criopreservación; formación de raíces; micropropagación; osmótico.

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Abbreviations used: BAP (benzyl amino purine), IAA (indole-3-acetic-acid), IBA (indole-3-butyric-acid), LN (liquid nitrogen), MS (Murashige and Skoog), NAA (naphthalene acetic acid), PVS2 (plant vitrification solution 2).

Introduction

Stevia rebaudiana is a perennial herb that belongs to the Asteraceae family. It is a natural sweetener plant known as «sweet weed», which is estimated to be 300 times sweeter than cane sugar (Liu and Li, 1995; Chalapathi and Thimmegowda, 1997; Savita *et al*., 2004). The seeds of *S. rebaudiana* show a very low germination percentage and do not produce uniform emergence and growth, resulting in great variability in plant growth and maturity and un-uniform plant sweetening levels and composition (Nakamura and Tamura, 1985; Sivaram and Mukundan, 2003). Vegetative propagation is limited by the lower number of individuals that can be obtained simultaneously from a single plant (Debnath, 2008). Due to the above-mentioned difficulties, tissue culture is the only alternative method for rapid propagation of *S. rebaudiana* plants. Plant tissue culture has some advantages over other propagation systems, as it provides rapid clonal propagation of uniform plants throughout the year (Durkovic and Misalova, 2008). Hence *in vitro* propagation appears as an alternative technique for rapid multiplication of stevia within a short span of time. Micropropagation of *S. rebaudiana* can be carried out through direct organogenesis and shoot multiplication provides a rapid reliable system for a production of large number of genetically uniform disease free plantlets, irrespective of the season. Although earlier attempts for micropropagation have been carried out, considerable effort is still required to make it more practical (Kornilova and Kalashnikova, 1997; Ahmed *et al*., 2007; Ibrahim *et al*., 2008).

Traditional vegetative plant germplasm conservation under natural condition methods suffers from many drawbacks. Plants are exposed to destructive biotic and abiotic stresses. Moreover, the maintenance of clonal orchards is labor intensive and expensive (Shibli *et al*., 2006). Slow growth culture, for medium-term preservation, and cryopreservation techniques, for long-term preservation, are two kinds of *in vitro* germplasm conservation methods which could offer practical means of germplasm storage of plant species (Shatnawi *et al*., 2007; Zarghami *et al*., 2008). Slow growth techniques are recommended for the storage of shoot cultures in many laboratories, as well as in many regional and international germplasm conservation centres (Shibli *et al*., 2006).

Cryopreservation is an attractive alternative for the preservation of plant material at ultra-low temperature (–196°C, *i.e.,* the temperature of liquid nitrogen), since under these conditions, biochemical and most physical processes are completely arrested. Such plant material can be stored for unlimited periods (Panis and Lambardi, 2005). Recently, some simplified and reliable cryogenic procedures have been developed such as vitrification method. Vitrification allows tissue survival by avoiding ice formation and can be achieved by increasing cell viscosity with exposure to highly concentrated cryoprotectants and dehydration followed by rapid cooling (Fang *et al*., 2004; Shatnawi *et al*., 2007). In theory, this method enables plant materials to be stored indefinitely without physiological alteration or generation of somaclonal variation, and it requires minimal storage space and maintenance (González-Benito *et al*., 2004; Wang *et al*., 2005). The key to successfully cryopreserve plant material, therefore, is the acquisition of osmotolerance, and the mitigation of any injurious effects during the dehydration process (Panis and Lambardi, 2005). Shoot tips are the preferred source of material, as they allow the conservation of a stable, physiologically differentiated tissue, rather than undifferentiated callus or meristem tissue alone (Shibli *et al*., 2006). To our knowledge, no research to date has been published on the *in vitro* conservation and cryogenic storage of *S. rebaudiana*. Therefore, this investigation was initiated to develop an efficient clonal propagation method as a primary step for preservation of *S. rebaudiana* for medium term (slow growth method) and long term conservation (cryopreservation) through vitrification.

Material and methods

Plant material and culture conditions

Microshoots of *S. rebaudiana* were obtained from the National Center for Agricultural Research and Extension (NCARE), Al-Baqa, Jordan. Healthy apical shoots were selected and trimmed to approximately 10 mm in length from 5-yr old mother plants. The leaflets were also removed prior to surface sterilization. Shoot tips were immersed in 70% ethanol for 30 s and placed in 1% NaOCl, together with two drops of Tween 20, shaking for 30 min. After sterilization, each explant was rinsed three times in sterile deionized water in a laminar flow cabinet. Explants were initially cultured on Murashige and Skoog (MS) medium (1962) supplemented with 0.5 mM myo-inositol, 0.34 mM thiamine HCl, 2.4 mM pyridoxine HCl, 4.1 mM nicotinic acid and 3% sucrose. The pH was adjusted to 5.8, and 7 g L^{-1}

agar agar was added prior to autoclaving. In each of 250 mL flask, 60 mL of medium were dispensed. The breather hole of each container was plugged with cotton wool to facilitate gas exchange and media were autoclaved for 20 min at 121°C. Explants were incubated at 24 ± 2°C with a 16 h photoperiod and irradiance of 50 μmol m^{-2} s^{-1} supplied by cool white fluorescent lamps. Shoots produced from these explants were subcultured after six weeks onto MS medium supplemented with 1.0 mg L^{-1} 6-benzylaminopurine (BAP) and 0.1 mg L^{-1} indole acetic acid (IAA), every 4 to 6 weeks to initiate enough plant material prior to experiment initiation.

Effect of cytokinins on shoot proliferation

Explants were subcultured onto the basal medium without growth regulators for 4 weeks, to avoid any carryover effect of the cytokinin. Microshoots with apical bud (15 mm in length) were then subcultured to fresh MS medium. For shoot proliferation, microshoots were subcultured to MS medium supplemented with either benzyl amino purine (BAP), kinetin or zeatin at 0.0, 0.5, 1.0, 1.5, 2.0 or 5.0 mg L^{-1}, with or without 0.2 mg L^{-1} indole-3-butyric acid (IBA). For each replicate, 60 mL of the medium were dispensed into 250 mL flask. Each treatment consisted of 20 replicates, each with 3 microshoots. Culture conditions were maintained as described above. After six weeks, data were collected on number of axillary shoots per initial explant and maximum shoot height.

Effect of auxins on root formation *in vitro*

Microshoots (15 mm in length) were subcultured on MS medium containing IBA, IAA or naphthalene acetic acid (NAA) at 0.0, 0.4, 0.8, 1.2, 1.6 or 2.0 mg L^{-1}. For each replicate, 60 mL of the medium was dispensed into 250 mL culture vessels. Each treatment consisted of 20 replicates, each with 3 microshoots. Culture conditions were identical to those described previously. Six weeks later, data were collected on shoot height, number of roots per initial explant, root length, and rooting percentage.

Ex vitro acclimatization

In vitro rooted plantlets were extracted from the medium, washed in a water bath at 35-40°C and transplanted to 6 × 6 × 6 cm plastic pots filled with sterile mixture of 1 peat:1 perlite. Shoots were grown under intermittent mist. Humidity was reduced gradually by making a hole on the plastic bag increasing its size every 3 days over 2-3 weeks. Data were collected for survival percentage of the acclimatized plants after six weeks.

Medium term conservation

Prior to the experiment, *in vitro* grown shoots were transferred to hormone-free MS medium for two weeks. Shoots of 15 mm length were then transferred to hormone-free MS medium supplemented with a series of concentrations (0, 3%, 6%, 9% or 12% w/v) of sucrose, sorbitol or mannitol. The stored explants were incubated in the growth room with 16 h photoperiod (50 μmol m^{-2} s^{-1}) or under dark conditions, at 24 ± 2°C. Survival data were collected after 16 and 32 weeks in storage. Data on regrowth were collected four weeks following subculture on hormone-free fresh MS medium.

Cryogenic storage by vitrification method

Shoot tips (2-3 mm) were excised under microscope from healthy *in vitro* shoots. The meristematic dome appeared as a glossy spot, surrounded by two or three leaf primordia. Excised shoot tips were precultured on solid MS medium supplemented with 0.4 M sorbitol and 0.1 M sucrose for 1 d in the dark. Precultured shoot tips were placed in cryotubes (10 tips per replicate) and loaded with 1.0 mL of the loading solution (80% plant vitrification solution 2 (PVS2); Sakai *et al*., 1990) for 20 min, at 0°C. To check the effect of loading solution on the growth of the shoot tips, subsequently shoot tips were cultured on MS medium with 3% sucrose, and incubated in the dark for one week, and then transferred to the growth chamber at 24 ± 2°C. The loading solution was then removed, and replaced with iced PVS2 which contained 30% (w/v) glycerol, 15% (w/v) DMSO, 15% ethylene glycol and 14% (w/v) sucrose dissolved in full strength MS medium. Shoot tips were exposed to the vitrification solution for varying times (0, 40, 80, 120, 160 or 200 min), at 0°C. The vitrification solution was replaced once at the end of the exposure time, and then the tips were transferred to 2 mL cryotubes and suspended in 0.5 mL of fresh PVS2 prior to immersion in liquid nitrogen (LN). Cryotubes containing the shoot tips were plunged directly in LN,

and maintained at –196°C for a period of 1 h to 1 d. After storage in LN, cryotubes were rewarmed rapidly in a water bath at 45ºC for 2 min. Subsequently, the vitrification solution was drained from the cryotubes and replaced with MS liquid medium supplemented with 14% (w/v) sucrose, in which the tips were washed for 15-20 min. Then shoot tips were transferred to solid hormone-free MS medium supplemented with 3% (w/v) sucrose. Cultures were then incubated in the dark for one week, and then transferred to the growth room conditions.

Regrowth was defined as the percentage of shoot tips resuming growth six weeks after plating out. Recovery of shoot tips was observed at weekly intervals. The young plantlets were subcultured on the optimal multiplication medium defined. Surviving plantlets were then subcultured into a hormone-free MS medium every 3 weeks. Approximately ten shoots were tested and each experiment was repeated six times.

Statistical analysis

Data were subjected to ANOVA analysis, and means with standard errors were obtained. Mean values were compared according to least significant differences test (LSD) at $p = 0.05$. Data were analyzed using STATISTICA (StatSoft, 1995).

Results

Effect of cytokinins on shoot proliferation

The addition of BAP (> 1 mg L^{-1}), kinetin or zeatin significantly increased shoot growth and proliferation of *S. rebaudiana*. Of the three cytokines tested, BAP at 1.5 mg L^{-1} was found to be the most effective plant growth regulator with regard to shoot proliferation (Table 1). Medium enriched with 1.0 to 2.0 mg L^{-1} kinetin showed maximum response; while zeatin concentrations from 0.5 to 2.0 mg L^{-1} significantly increased shoot proliferation and maximum shoot lengths were obtained on media with 1.0 to 2.0 mg L^{-1} zeatin.

Effects of auxin on root formation *in vitro*

Root formation did not occur in the absence of auxins. Auxins increased rooting percentage at all tested concentrations (Table 2). Thus, 90% of the shoots rooted on MS medium supplemented with 0.4 mg L^{-1} of IBA, IAA or NAA, while at 2.0 mg L^{-1} of all auxins 100% of the shoot were successfully rooted. Maximum shoot length (61 mm) was recorded at 1.2 mg L^{-1} NAA. Maximum root number (16 root/ex-plant) was formed at 1.2 mg L^{-1} IBA, and maximum root length (65.9 mm) at 0.8 mg L^{-1} IAA.

Table 1. Effect of different benzyl amino purine (BAP), kinetin or zeatin concentrations on shoot length and axillary shoot number of *Stevia rebaudiana* after six weeks on culture

Growth regulators (mg L^{-1})	Shoot length (mm)	Number of axillary shoots/explant
BAP		
0.0	28^{c}	1.6^{d}
0.5	30^{c}	4.6^{b}
1.0	30^{c}	4.7^{b}
1.5	44^{b}	8.9^{a}
2.0	55^{a}	5.2^{b}
Kinetin		
0.5	30^{c}	1.7^{d}
1.0	45^{b}	3.2^{c}
1.5	45^{b}	3.5^{c}
2.0	44b	3.5^{c}
Zeatin		
0.5	30^{c}	2.9^{c}
1.0	41^{b}	3.4^{c}
1.5	46^{b}	4.6^{b}
2.0	47^{b}	4.4^{b}

Mean values within a column with same letter are not significantly different based on least significant difference (LSD) at $p = 0.05$. Each treatment consisted of 20 replicates, with three microshoots in each. Experiments were repeated twice. Medium supplement with 0.2 mg L^{-1} IBA.

Ex vitro acclimatization

Approximately 90% of the plantlets survived *ex vitro* acclimation and no variation was observed among the plantlets, which produced good healthy plants.

Medium term conservation

The addition of sucrose to the medium increased survival of microshoots as compared with the control (without sucrose), when cultures were stored for 16 or 32 weeks. A total of 94.6% survival was obtained when cultures were stored on medium supplemented with

Table 2. Effect of indole-3- butyric acid (IBA), indole acetic acid (IAA) and naphthalene acetic acid (NAA) concentrations on number of shoots, shoot length, number of roots, root length and formation of *in vitro* shoots of *Stevia rebaudiana* after six weeks on culture

Growth regulator (mg L^{-1})	Number of shoots/explant	Shoot length (mm)	Number of roots/explant	Root length (mm)	Root formation (%)
IBA					
0.0	1.20[a]	25.30[d]	0.00[d]	0.00[c]	0.0
0.4	1.40[a]	48.75[b]	8.24[c]	44.5[b]	90
0.8	1.30[a]	49.70[b]	13.56[b]	43.4[b]	90
1.2	1.35[a]	59.80[a]	15.78[a]	45.8[b]	95
1.6	1.35[a]	47.80[b]	14.84[a]	39.3[b]	90
2.0	1.35[a]	57.41[a]	9.80[c]	38.9[b]	100
IAA					
0.4	1.40[a]	40.30[c]	6.34[c]	54.5[a]	90
0.8	1.40[a]	48.40[b]	8.45[c]	65.9[a]	90
1.2	1.35[a]	48.76[b]	7.96[c]	57.7[a]	100
1.6	1.25[a]	44.41[c]	6.20[c]	59.7[a]	100
2.0	1.30[a]	44.58[c]	7.25[c]	57.8[a]	100
NAA					
0.4	1.30[a]	38.57[c]	9.24[c]	43.5[b]	90
0.8	1.35[a]	50.67[b]	8.30[c]	45.2[b]	95
1.2	1.35[a]	60.85[a]	7.27[c]	55.83[a]	95
1.6	1.40[a]	57.70[b]	3.30[c]	56.7[a]	95
2.0	1.40[a]	52.38[b]	3.35[c]	45.8[b]	100

Mean values within a column with same letter are not significantly different based on least significant difference (LSD) at $p = 0.05$. Each treatment consisted of 20 replicates, with three microshoots in each. Experiments were repeated twice.

3% sucrose after 16 weeks under light conditions, while 93.6% were able to survive after 32 weeks. Moreover, with that concentration 89.3% of the shoots were able to growth after 32 week storage when stored under light conditions (Table 3). Under dark conditions, survival was reduced significantly after 32 weeks on all media supplemented with sucrose, sorbitol or mannitol. Highest sucrose, sorbitol or mannitol level (12%), usually led to a significant decrease in survival, compared with the 3-9% sucrose treatments in both light and dark conditions. The best treatment in dark condition was 3% sorbitol with 61.8% survival after 32 weeks and a 36.6% of regrowth (Table 3).

Cryogenic storage

Shoot tips were precultured on solid MS medium supplemented with 0.4 M sorbitol and 0.1 M sucrose for one day in the dark to initiate acclimation to osmotolerance. Shoot tips regrowth decreased significantly with increased exposure time to any of the vitrification solutions tested (data not shown). However, the percentage of shoot formation of unfrozen shoot tips was generally higher than those of cryopreserved tips. The percentage of shoot formation after cryopreservation using PVS2 ranged from 15 to 66%. For unfrozen shoot tips 90% regrowth was obtained following 40-60 min of exposure to PVS2, while a regrowth percentage of 13% was obtained after exposure for 150 min. Cryopreserved tips treated with PVS2 for 40 min showed 30% regrowth and the maximum of regrowth was obtained with 60 min of exposure. Longer exposures to PVS2 for 150-200 min caused significant reduction in regrowth for frozen and unfrozen tips (Fig. 1). Frozen shoot tips, once thawed, developed into shoots, after rapid thawing in a water-bath at 45°C. It was found that using a loading solution was essential to allow shoot formation after storage in LN. Shoot formation of vitrified tips increased gradually with the duration of exposure to PVS2, reaching a maximum value (66%) after 60 min of exposure.

Table 3. Effect of different concentrations of sucrose, sorbitol or mannitol on survival and regrowth percentage of *in vitro* plantlets with shoot tips of *Stevia rebaudiana* stored under light/dark conditions at 24 ± 2°C after 16 or 32 weeks

Osmotic agents % (w/v)	Sucrose			Sorbitol			Mannitol		
	Survival after-16 weeks (%)	Suvival after-32 weeks (%)	Regrowth after-32 weeks (%)	Survival after-16 weeks (%)	Suvival after-32 weeks (%)	Regrowth after-32 weeks (%)	Survival after-16 weeks (%)	Suvival after-32 weeks (%)	Regrowth after-32 weeks (%)
Light condition									
0	0.0^{e}	0.0^{f}	0.0^{d}	0.0^{d}	0.0^{c}	0.0^{b}	0.0^{d}	0.0^{c}	0.0^{e}
3	94.6^{a}	93.6^{a}	89.3^{a}	76.6^{a}	65.0^{a}	48.3^{a}	58.3^{a}	48.3^{ab}	33.3^{b}
6	65.3^{b}	60.0^{bc}	41.6^{b}	73.6^{b}	66.6^{a}	31.6^{a}	58.3^{a}	48.3^{ab}	46.6^{a}
9	53.3^{bc}	36.6^{de}	32.3^{c}	81.6^{a}	0.0^{c}	0.0^{b}	52.0^{ab}	51.6.0^{a}	20.0^{bc}
12	41.6^{cd}	30.0d^{e}	22.6^{c}	49.6^{b}	0.0^{c}	0.0^{b}	38.3^{bc}	3.6^{c}	11.7^{d}
Dark condition									
0	0.0^{e}	0.0^{a}	0.0^{d}	0.0^{d}	0.0^{c}	0.0^{c}	0.0^{d}	0.0^{c}	0.0^{e}
3	68.3^{b}	46.6^{cd}	47.6^{b}	65.6^{bc}	61.7^{a}	36.6^{a}	28.3^{c}	23.3^{b}	11.6^{c}
6	55.0^{bc}	43.3^{cd}	44.3^{b}	62.6^{bc}	50.0^{a}	30.0^{a}	51.6^{ab}	33.3^{ab}	15.0^{bc}
9	55.0^{bc}	48.3^{cd}	33.3^{c}	70.6^{a}	35.0^{b}	13.3^{b}	41.6^{bc}	25.2^{bc}	6.7^{de}
12	25.0^{d}	5.6^{f}	4.6^{d}	44.6^{bc}	11.6^{c}	0.0^{b}	35.0^{bc}	0.0^{c}	0.0^{e}

Mean values within a column with same letter are not significantly different based on least significant difference (LSD) at $p = 0.05$. Each treatment consisted of 20 replicates, with three microshoots in each. Experiments were repeated twice.

Discussion

The addition of BAP, kinetin or zeatin increased the formation of new shoots/explant and shoot length after six week growth period. This result was similar to previous findings on *Sesbania rostrata* (Jha *et al*., 2002). BAP at 1.5 mg L^{-1} was found to be the most effective concentration for formation of new shoots. This is similar to a previous study on *Stevia rebaudiana* (Ahmed *et al*., 2007). In the present study, BAP proved to be useful for micropropagation for this species because of its tremendous ability to induce shoot formation. These results were in agreement with those found by Mitra and Pal (2007) and Anabzhagan *et al*. (2010) in *S. rebaudiana*.

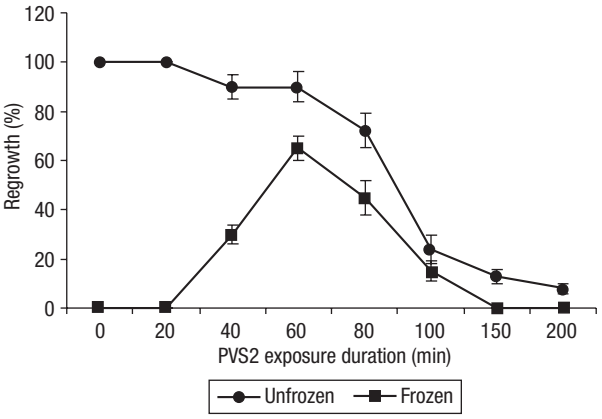

Figure 1. Effect of exposure time with plant virification solution 2, at 0°C, on regrowth percentage of unfrozen and frozen tips of *Stevia rebaudiana.* Approximately 10 shoot tips were tested for each replicate. Experiments were repeated twice. Regrowth was defined as the percentage of cryopreserved shoot tips resuming normal growth six weeks after plating out. Error bars represent standard error of means.

Enhanced rooting was obtained in a range of treatments, whereas no rooting was found in the control. With the supplement of auxin to the media, mostly single shoots were produced. However, the use of 2.0 mg L^{-1} of IBA, IAA or NAA produced large quantities of calluses at the base of the microshoots. This was similar to previous findings by Armstrong and Johnson (2003) in *Ceratopetalium gummiferum.* Debnath (2008) reported that *S. rebaudiana* was rooted on MS medium supplemented with 2.0 mg L^{-1} IBA. Low concentrations of IBA, IAA and NAA were effective in inducing rooting in *S. rebaundiana,* and the development of roots was quite similar at the different concentrations. These findings are compatible with those of Mitra and Pal (2007) and Anabazhagan *et al*. (2010) in *S. rebaundiana*.

Sucrose, sorbitol or mannitol were used as an osmotic agent and were able to play an important role in the conservation as explants of *S. rebaudiana*, compared with the controls. Bertrand-Desbrunais *et al.*

(1992) found that elongation of *in vitro* stored shoot tips of C*offea* spp. was maximized at a sucrose concentration of 2%, and shoot tip elongation decreased as the sucrose concentration was increased in the medium. Assy-Bah and Engelmann (1992) reported that sucrose could be used for the conservation of zygotic embryos of *Cocos nucifera. In vitro* stored *S. rebaudiana* explants showed a poor growth response when cultures were stored at sucrose concentrations higher than 3%, for either 16 or 32 weeks, under either light or dark conditions. Similar results were obtained by Brown *et al*. (1979) with tobacco callus cultures when sucrose level was higher than 3%. Bertrand-Desbrunais *et al.* (1992) reported that increased sucrose levels could be expected to reduce cell metabolic activity. Certainly, no new shoots were obtained during storage in treatments containing sucrose, sorbitol, or mannitol higher than 6%. However, this could also have been directly due to the absence of required hormones, as the medium used was hormone-free to avoid any callus formation after storage. In addition, the fact that no roots developed during the storage period could be due to the absence of required hormones (auxin). Orlikawoska (1992) indicated that elimination of BAP from the medium prevented shoot elongation. Thus, the need for growth regulators under these conditions is not clear, and may depend on the hormonal balance and requirements of the particular species or genotype.

The development of techniques to successfully store shoot tips in LN is needed for conservation of *S. rebaudiana*. For successful cryopreservation, it is vital to dehydrate cells sufficiently for survival after cooling in LN through vitrification. It was found that using loading solution was essential to allow shoot formation. A similar finding was reported by Takagi *et al*. (1997), preserving tips of *Colocasia esculenta*. Increasing time of exposure to vitrification solutions leads to increase solute concentrations inside the plant tissue (Bachiri *et al*., 1995). When shoot tips of this species were subjected to a vitrification solution for 0 or 40 min, without immersion in LN, shoot formation was very high. However, longer exposure to vitrification solution reduced significantly shoot tip regrowth. The highest regrowth rates of cryopreserved shoot tips (66%) was obtained after exposure to PVS2 for 60 min at 0°C. Sakai *et al.* (1990) demonstrated that complete vitrification of the cryopreserved plant tissues avoids by intra and extracellular ice crystallization. Thus, the optimal exposure duration proved to have critical effect on shoot formation after freezing in LN. Results obtained in this study suggest that long-term storage of shoot tips is possible, using the cryogenic methods developed. Hormone-free MS medium was used in this study; in a successful effort to avoid any callus formation, and preserve true-to-type regrowth. Plantlets surviving cryopreservation were subcultured on MS medium without growth regulators for 2 weeks, and then placed on the multiplication medium found to be optimal. The media successfully induced new shoot in all treatments.

Cryopreserved shoot tips were generally regenerated into normal plantlets after thawing, and continuous subculture of surviving shoot tips was employed to obtain healthy shoots. In this study, cryopreserved shoot tips produced shoots identical in appearance with those of the controls, and plants regenerated from vitrified shoot tips were morphologically uniform. The methods developed in this study appear to be suitable for cryopreservation of shoot tips from *in vitro* cultures of *S. rebaudiana*. Additional studies are still needed to increase recovery of tips after cryopreservation, and to standardize the protocols and evaluate genetic stability of cryogenic stored plant tissues.

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