Bio Bulletin 3(1): 30-38(2017)
(Published by Research Trend, Website: www.biobulletin.com)

ISSN NO. (Print): 2454-7913
ISSN NO. (Online): 2454-7921

In Vitro Storage of *Stevia rebaudiana* Bertoni under Slow Growth Conditions and Mass Multiplication after Storage

Ely Zayova, Trendafil Nedev, and Ludmila Dimitrova
Department of Applied Genetics and Plant Biotechnology, Institute of Plant Physiology and Genetics, Acad. G. Bonchev Street, Bldg. 21, 1113 Sofia, Bulgaria

(Corresponding author: Ely Zayova, e_l_y@abv.bg)
(Published by Research Trend, Website: www.biobulletin.com)
(Received 27 December 2016; Accepted 02 February 2017)

ABSTRACT: *Stevia rebaudiana* Bertoni (Asteraceae) is a valuable medicinal plant widely used in the pharmaceutical and food industry. An efficient protocol for in vitro storage of *S. rebaudiana* plants under slow growth conditions was established. Slow growth cultures were obtained on half strength Murashige and Skoog basal medium (½ MS) supplemented with the osmotic agent mannitol (0; 1; 2; 3 and 4% w/v) for three and six months without any subculture. The 3% mannitol reduced the growth of *S. rebaudiana* plants in terms of plant height, root length and number of roots formed under low light intensity. The all studied growth parameters decreased significantly with the increase of the storage period. Cultures stored at 22±1 °C did not survive more than six months. The conserved plants were retrieved on the ½ MS basal medium. The plants storaged for three months multiplied more rapidly on propagation medium with 1 mg L^{-1} 6-benzylaminopurine (BAP) than these conserved for six months. The micropropagated plants were successfully in vitro rooted and ex vitro **acclimatizated. They showed very good growth and development under field conditions.**

Keywords: in vitro cultured; mannitol; slow growth; micropropagation

INTRODUCTION

Stevia rebaudiana Bertoni (*Asteraceae*) is a perennial shrub of South America, a source of low calorie natural sweeteners, which main components are the diterpene glycosides (stevioside and rebaudioside A). The leaf extracts exhibit various biological activities (antibacterial, antimicrobial, antifungal, antidiabetic, antioxidant, and anticancer). At present, stevia is grown commercially and used as natural sweetener in many parts of Brazil, Paraguay, Japan, China, United States, Canada, and Europe. In Bulgaria, there is great interest in the stevia cultivation, although the cold environmental conditions allow it to grow only as an annual plant, since its rhizomes cannot survive at low soil temperatures during winter. The *traditional* propagation methods of *S. rebaudiana* are *slow* and unreliable.

Poor seed germination of *S. rebaudiana* is one of the factors limiting large-scale cultivation (Felippe and Lucas, 1971). Significant variations in the sweetening level and composition were observed, when the propagation is carried out by seeds (Sivaram and Mukundan, 2003). Therefore, the vegetative propagation is preferred, although it is limited by the low number of individuals obtained from a single plant (Jain *et al*., 2009; Mehta *et al.*, 2012). Successful clonal micropropagation of *S. rebaudiana* is a primary step for in vitro conservation. *However, preservation by micropropagation is not reasonable due to the requirement of frequent subculture.* There are two in vitro techniques for conservation of medicinal species: slow growth procedures and cryopreservation. Slow growth procedures provide short- and medium-term storage options, while cryopreservation enables long-term storage of the

plant material (Shibli *et al.,* 2006; Kasagana and Karumuri, 2011). Slow growth techniques, based on modification of the culture media *with supplements of osmotic agents* as sucrose, mannitol and sorbitol (Engelmann, 2011)*, growth inhibitors* (Srivastava *et al.,* 2013), or the manipulation of culture conditions by low temperature (Negri *et al.,* 2000; Joshi and Kumar, 2013) are used. The most effective way to extend the periods between subcultures is combining of osmoticums with low temperature and low light intensity (Ammara *et al.,* 2014). The application of these in vitro conservation techniques allows cultures to remain viable with a slow growth rate and a significant increase in the storage life of the tissues (*Rao,* 2004; Cruz-Cruz *et al.,* 2013). In recent years, different biotechnological approaches are used to conserve endangered and rare medicinal species, providing conservation of pathogen free material and biodiversity. The supplementing of mannitol or sorbitol to the culture medium effectively decreases the growth of different plant species. These osmoticums reduce mineral uptake by the cell through differences in osmotic pressures thereby retarding plant growth (Thompson *et al.,* 1986). During prolonged in vitro storage physiological changes or genetic variation may occur, and there is a risk for the genetic stability of the conserved plant materials (Cruz-Cruz *et al.,* 2013). On the other hand, cryopreservation by vitrification also could be useful for long-term storage of *S. rebaudiana* germplasm (Shatnawi *et al.,* 2011). Also, there is a need of the detailed knowledge of the chemical profile of secondary metabolites, especially for plants originated from in vitro cultures. Lata *et al.* (2014) reported there are no significant differences in rebaudioside A and stevioside content between the mother and re-grown *S. rebaudiana* plants following in vitro storage. There are a number of publications describing successful shoot induction and micropropagation of *S. rebaudiana* (Ibrahim *et al*., 2008; Das *et al*., 2011; Ghauri *et al.,* 2013; Razak *et al*., 2014), but information on in vitro storage with an osmotic agent for reducing growth is limited in the available literature.

The aim of this study was to develop a protocol for in vitro conservation of *S. rebaudiana* under slow growth conditions and mass micropropagation after the storage period.

MATERIALS and METHODS

A. Sample collection

The initial in vitro cultures of *S*. *rebaudiana* were obtained from aseptic seedlings earlier (Zayova *et al.*, 2013). The seeds of the plant species were purchased from the commercial seed source Company "Pase Seeds" SKU: N/A, USA (Lot 00318G63423014). The actively growing plants dissected from 2-month-old in vitro culture were used as a started plant material for slow growth storage.

B. Experimental procedures

In vitro storage by slow growth. Prior to the experiment, in vitro grown plants of *S. rebaudiana* were transferred to half strength Murashige and Skoog, 1962 basal medium (½ MS) containing 20 g L^{-1} sucrose, 7 g L^{-1} agar and 0.1 mg L^{-1} indole-3-butyric acid (IBA) for two weeks in order to align the phase of growth and development. The effect of the osmotic agent on the survival and growth of in vitro cultured plants was observed using the same medium supplemented with mannitol at different concentrations (0, 1, 2, 3 and 4% w/v). For all experiments, two plants were put individually in glass tubes (20 × 150 mm), with 10 ml of medium. Each treatment involved 40 plants. The first subculturing was carried out after three months. However, part of the cultured plants was left on the same medium for additional three months (a total of 6 months without subculturing). The survival of the storaged in vitro cultures was determined by the presence of green plants with healthy growing tips without necrosis after one, three and six storage months. Various growth parameters like, percentage of rooted plants, plant height, root length, and number of roots at the end of the third and sixth months of storage were recorded. Data for control plants was collected after one month under storage conditions.

Plants recovery and shoot multiplication after storage. The plants that had survived the osmotic stress (3% mannitol) for three or six months were subcultured on fresh ½ MS basal medium without mannitol for two passages of four weeks each under *standard* culture conditions for recovery after in vitro storage. *After this period, survival percentage was recorded as a measure of plant recovery.* For *micropropagation*, the shoot tips and nodal segments from plants, recovered after two storage periods, were cultured on full strength MS media with vitamins, 3% w/v sucrose, and 0.7% w/v agar supplemented with 6-benzylaminopurine (BAP) at three defferent concentrations (0.5, 1.0 and 2.0 mg L^{-1}). Each treatment consisted of 20 explants. Four weeks later, the data on the number of shoots per initial explant were collected*.*

In vitro rooting and ex vitro acclimatization after storage. For root induction of the multiplied microshoots, the same ½ MS basal medium was used (½ MS0 control medium without auxin and ½

MS1 medium supplemented with 0.1 mg L^{-1} IBA). Two shoots per culture tube were cultured vertically in a glass tube (150 x 20 mm), containing 8 ml of rooting medium. Each treatment involved 40 plants. Three weeks later, data on rooting percentage, shoot height, number of roots per plant, and root length were collected. For acclimatization under ex vitro conditions, the rooted plants were carefully taken out from the vessels and washed under running tap water to remove the gelling agent. They were transplanted to small plastic pots (8 cm diameter) containing different mixtures: 1) soil: perlite: coco substrate (1:1:1 v/v/v); 2) soil: perlite: coco substrate (2:1:1 v/v/v); 3) soil: sand: perlite (1:1:1 v/v/v) and 4) soil: sand: perlite (2:1:1 v/v/v). The potted plants were covered with a transparent polythene membrane to ensure high humidity (90%) and were opened after two weeks. Data were collected for survival percentage of the acclimatized plants (20 individuals per treatment) after five weeks. After two months of ex vitro acclimatization, the plants were transferred to greenhouse for further growth and development for a period of three weeks. Finally, the plants were planted in field conditions.

Culture conditions. The pH of the media was adjusted to 5.7±0.1 with 1 N NaOH or 1 N HCl, before adding the gelling agent (agar, 7 g L^{-1}) and autoclaving for 20 min at 121°C. All the chemicals used for preparing the media were purchased from *Duchefa Biochemie B.V.* The stored plants were maintained in a growth room with 16 hour photoperiod (20 µmol $m^{-2}s^{-1}$) at 22±1°C. The recovered in vitro cultures were kept in the growth room at normal conditions (22±1 °C under a 16 hour photoperiod using Philips cool white fluorescent tubes of 40 µmol $m^{-2}s^{-1}$ light intensity). Ex vitro acclimatization was carried out at 24±1 °C and 70% relative humidity under 16 h illuminations (50 µmol m^{-2} s^{-1}).

C. Statistical analysis

Each experiment was repeated twice. Data were subjected to one-way ANOVA analysis of variance for comparison of means, and significant differences were calculated according to Fisher LSD test at the 5% level using a statistical software package (Statigraphics Plus, version 5.1 for Windows). Data were reported as means ± standard error.

RESULTS and DISCUSSION

A. In vitro storage by slow growth

The effect of different mannitol concentrations added to the conservation medium on the survival percentage of *S. rebaudiana* plants was evaluated after three and six months of storage (Table 1). Microplant survival was 100% on ½ MS medium without osmotic agent for the first month. In the control medium, the cultivated plants grew faster and fill glass tube (Fig. 1a). After one month of culture, the plants had to be transferred to fresh medium due to the exhaustion of nutrients. Maximum percentage of plant survival (80%) was found in ½ MS with 1% mannitol followed by 60% in the same medium with 2% mannitol at the end of the third month (Table 1).

Fig. 1. Effect of the mannitol concentration on the plant growth of *S. rebaudiana*: (a) control plants after one month of cultivation; (b) plants cultured on MS medium with 3% mannitol after three storage months; (c) plants cultured on MS medium with 3% mannitol after six storage months.

Table 1: Survival percentage of *S. rebaudiana* plants during slow growth storage.

Treatments	Survival, %		
	1 Month	3 Months	6 Months
½ MS, control	100	-	-
1% *mannitol*	100	80	65
2% *mannitol*	80	60	50
3% *mannitol*	65	55	40
4% *mannitol*	25	20	-

After six months of culture, the survival percentage decreased (65% and 50% respectively on the medium with 1% or 2% mannitol), but the plant growth was not yet reduced. Inhibition of microplant growth up to 40% survival was achieved by adding 3% mannitol to the conservation medium. All plants were with small leaves and short stems. The survival percentage of the plants cultivated on the medium with 4% mannitol was low (only 20%) at the end of the third month and the plants died at the end of the sixth storage month. The high concentration of osmotic agent was deleterious for plant survival. The storage period significantly affected the survival percentage and plant growth. Increasing the conservation period of plants up to six months decreased their survival percentage compared with the same parameter of those conserved for three months. The plant height, root length, and number of roots per plant also depended on the mannitol concentration. The growth of the plants, cultured on ½ MS basal medium containing mannitol was suppressed when compared with the control plants. The data presented in Table 2 showed the effect of mannitol concentrations on the growth parameters after three and six months under storage conditions.

The rhizogenesis decreased with the increase of the mannitol concentration. Maximum percentage of plant rooting (80%) was recorded on ½ MS medium with 1% mannitol, while root induction was 40% in the same medium with 3% mannitol at the end of third month. The presence of 4% mannitol in the medium blocked the growth and development of the plants and the roots at the end of the two storage periods. The number of the roots per plant significantly decreased when the osmotic concentration increased. Also, reduction in root length occurred with the addition of mannitol in the slow growth culture medium. There were significant differences of the average root length between the various treatments. The highest root length was recorded with 1% mannitol (2.4 cm), whereas 3% mannitol provided the lowest value (1.1 cm) after three months under storage conditions. A similar trend was observed for the root length which significantly was reduced after six storage months (Table 2). The plants cultured on the conservation medium with 3% mannitol for three months, formed a well developed root system. They had long main root with numerous secondary roots (Fig. 1b), while the plants cultured on the same medium for six months had very short roots (Fig. 1c).

Table 2: Effect of mannitol concentrations on growth parameters of *S. rebaudiana* under storage conditions.

Mannitol concentration	**Rooting, %**	**Plant height, cm**	**Root number per plant**	**Root length, cm**
½ MS, control	100	10.3 ± 0.47^{d}	6.2 ± 0.32^{d}	2.7 ± 0.21^{c}
After three months under storage conditions				
1%	80	4.3 ± 0.38^{c}	4.0 ± 0.31^{c}	2.4 ± 0.21^{c}
2%	60	2.8 ± 0.25^{b}	2.2 ± 0.26^{b}	1.6 ± 0.11^{b}
3%	40	1.4 ± 0.14^{a}	0.9 ± 0.12^{a}	1.1 ± 0.05^{a}
4%	-	0.9 ± 0.07^{a}	-	-
½ MS, control	100	10.3 ± 0.47^{c}	6.2 ± 0.32^{d}	2.7 ± 0.21^{d}
After six months under storage conditions				
1%	70	2.1 ± 0.22^{b}	2.9 ± 0.27^{c}	1.8 ± 0.17^{c}
2%	55	1.7 ± 0.16^{b}	1.5 ± 0.17^{b}	1.4 ± 0.12^{b}
3%	30	0.8 ± 0.07^{a}	0.5 ± 0.11^{a}	0.4 ± 0.05^{a}
4%	-	0.6 ± 0.05^{a}	-	-

Data are presented as means of 40 plants per treatment ± standard error. Different letters indicate significant differences assessed by the Fisher LSD test ($P \leq 0.05$) after performing ANOVA multifactor analysis.

Rhizogenesis was not recorded on the culture medium with 4% mannitol. The main requirement for in vitro storage is an effective method for micropropagation of *S. rebaudiana*, as described previously (Zayova *et al.,* 2013). However, every culture stage of this process requires a monthly transfer of plants to fresh culture medium. The main goal of the slow growth procedure is to extend the subculturing interval from one month to a period of several months. The reduction of the plant growth was achieved by modification of the culture medium. The osmotic agent mannitol significantly affected survival and growth of *S. rebaudiana* plants. The addition of 3% mannitol to the medium had proved efficient for reducing plant growth and subcultures frequency. However, the microplant survival did not surpass 40% and practically ceased at 4% mannitol. The reduction of MS salts and exposure of plants to low light intensity also had a beneficial effect on slowing the plant growth. The percentage of plant survival decreased rapidly with the increase in storage period, which may be due to desiccation and nutrient depletion of the media that proved detrimental during storage. On the other hand, the high mannitol concentration was harmful and caused plant death. The lethal concentration seemed to be species dependent (Silva and Scherwinski-Pereira, 2011). The slowing down of cell metabolism was a consequence of shoot growth under the slow growth conditions. Mannitol often has been supplemented to nutrient media to mimic osmotic stress, which causes reduction in growth. Slow growth in vitro storage was reported in many plant species such as enset (Negash *et al.,* 2001); rose (Charoensub and Phansiri, 2004); garlic cultivars (Hassan *et al.,* 2007), *Veronica multifida* ssp. (Holobiuc *et al.,* 2008), strawberry (Hassan and Bekheet, 2008), *Artemisia herba-alba* (Sharaf *et al.,* 2012). The highest rate of regrowth and survival of *S. rebaudiana* was observed at 2% sorbitol followed by 2% sorbitol with 2% dextrose (Lata *et al.,* 2014). In the current study, in vitro cultures of *S. rebaudiana* were maintained for a maximum of six months without subculture on slow growth medium (½ MS basal medium supplemented with 3% mannitol) at temperature 22±1ºC under low light intensity (20 µM $m^{-2}s^{-1}$). Also, extending the subculture interval to six months resulted in a significant decrease of plant growth parameters. The mean root number and root length decreased rapidly at the end of the six months due to depletion of the medium.

B. Plants recovery and shoot multiplication after storage

When the plants that had survived the osmotic stress from 3% mannitol were transferred on ½ MS medium without osmotic agent at standard growth conditions at the end of each conservation period, they return towards normal growth and development after two subcultivations. The survival percentage was dependent on the storage period. The plants conserved for three months were able to survive (100%) when recultured on normal growth medium. This percentage reduced to 60% when plants were conserved for six months. During recovery, the plants grow quickly and filled the culture vessel. The leaf browning disappeared. For multiplication, the recovered plants from the two storage periods were cut into tip and nodal segments. The results for the shoot tip culture of *S. rebaudiana* on MS medium with BAP (0.5; 1.0 and 2.0 mg L^{-1}) are shown in Fig. 2. The number of shoots varied with the concentration of the cytokinin used. The MS medium containing 1.0 mg L^{-1} BAP induced maximum number of shoots (2.6 and 1.9 shoots/ explant after the two storage periods, respectively within four weeks of culture (Fig. 3a). When the concentration of BAP was increased up to 2.0 mg L^{-1} the shoot number was found to be decreased. Also, several plant subcultivations were necessary for the micropropagation process. The propagation potential increased when the plants were continuously subcultured on the same propagation medium. The micropropagation results indicated a significant difference in the viability between the two storage intervals. The plant conserved for three months multiplied more rapidly on MS medium with 1 mg L^{-1} BAP under normal growth conditions than those storaged for six month. In contrast, the nodal segments grew in height and no new adventive shoots were formed at all tested BAP concentrations.

The shoot number of *S. rebaudiana* depended on the BAP supplemented to the culture medium. The stored plants can be micropropagated rapidly when needed. The presence of BAP alone or in combination with other plant growth regulators was essential for shoot multiplication of *S. rebaudiana* (Hossain *et al.,* 2008; Das *et al.,* 2011; Laribi *et al.,* 2012).

In our study, in vitro cultures which were maintained for three months under slow growth conditions at 3% mannitol generate new shoots in the first passage.

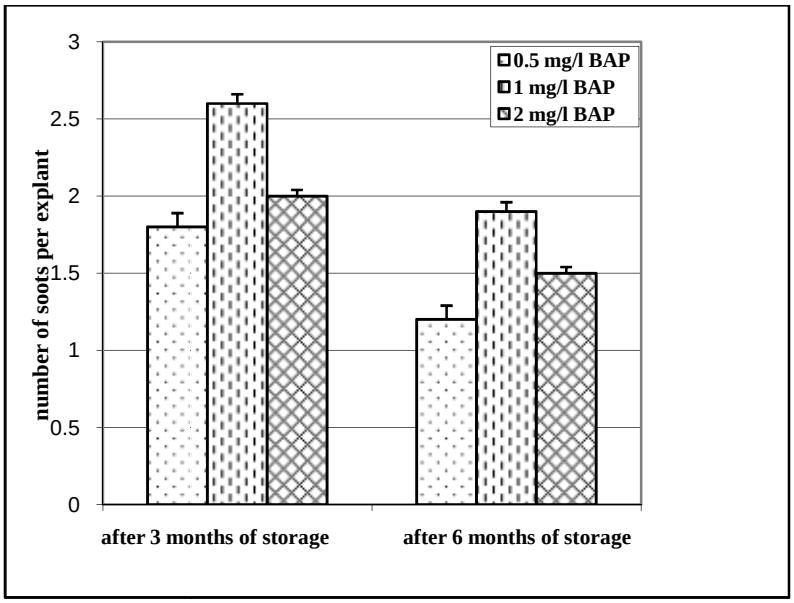

Fig. 2. Micropropagation ability of *S. rebaudiana* plants after storage at 3% mannitol for three and six months, recultured on MS medium with BAP for four weeks.

Fig. 3. Micropropagation of *S. rebaudiana* after storage: a) multiplicated plant; b) in vitro rooted plants; c) ex vitro acllimatezed plants; d) cultivated plants under field conditions.

Shoots, which were stored for six months under slow growth conditions multiplied very slowly and the multiplication process started after two or three subcultivations on the optimal MS propagation medium.

C. In vitro rooting and ex vitro acclimatization after storage

The propagated shoots were rooted on ½ MS medium containing auxin IBA (Table 3). Roots were observed within 12 days after the transfer to the rooting medium. Auxin free ½ MS medium (control) induced relatively few roots; therefore it needs the presence of auxin. Hall strenght MS medium with 0.1 mg L^{-1} IBA was very effective for the root growth. Significant differences between the percentage of in vitro rooted plants and the root number per plant on the control and IBA enriched medium were recorded after three weeks of culture. In addition, 0.1 mg L^{-1} IBA in the ½ MS medium stimulated rooting (80%) as compared to the control (20%) and gave a greater root number per shoot (3.8 with 1.6 cm versus 1.1 with 0.7 cm) for the propagated plants kept for three storage months. The root formation and number of roots per plant were significantly lower on the same media for the propagated plants kept for six storage months (Table 3). All the rooted plants were stable with very good leaf development and profuse roots, firmly fixed to the base of the stem (Fig. 3b). It is a guarantee for good ex vitro acclimatization. The results showed that ½ MS medium containing 0.1 mg L^{-1} IBA could induce roots within three weeks indicating that auxins enhanced root formation. Superiority of IBA over other auxins in root induction of *S. rebaudiana* also has been reported (Tadhani *et al.,* 2008; Mehta *et al*., 2012; Ghauri *et al.,* 2013).

Table 3: Rhizogenesis of *S. rebaudiana* propagated plants after *in vitro* storage.

Treatment		Rooted plants, %	Root number per plant	Root length, cm
Medium	**IBA, mg/l**			
After three months under storage conditions				
½ MS0	0	20	1.1±0.14[a]	0.7±0.11[a]
½ MS1	0.1	80	3.8±0.42[b]	1.6±0.21[b]
After six months under storage conditions				
½ MS0	0	10	0.6±0.09[a]	0.4±0.05[a]
½ MS1	0.1	65	1.3±0.19[b]	0.8±0.12[b]

Data are presented as means of 20 plants per treatment ± standard error. Different letters indicate significant differences assessed by the Fisher LSD test ($P \leq 0.05$) after performing ANOVA multifactor analysis.

The survival of *S. rebaudiana* plants after five weeks under ex vitro conditions was evaluated (Table 4). Initially, the rooted plants were transferred to plastic pots with different mixture substrates in a growth chamber and kept covered with polyethylene membranes for two weeks. The high levels of relative humidity enhanced the initial survival of the potted plants. Then, the membranes were removed and the survival of the plant material was scored under 70% humidity during next weeks. The tested mixtures had beneficial effect on the plants survival and growth. The highest percentage of plant survival was obtained after transplantation to soil, perlite and sand (1:1:1 v/v/v). Positive effect of this mixture on plant growth and development was observed during the first week after planting. At the acclimatization stage, the survival percentage was 95% and 70% respectively, for the microplants derived from both storage periods. During the adaptation, the plants continue to grow, which is a sign of successful ex vitro acclimatization (Fig. 3c). The plants were successfully hardened and transferred to greenhouse conditions.

Table 4: Ex vitro acclimatization of *S. rebaudiana* plants after in vitro storage.

Substrate type	Survival, %	
	Adapted plants after three storage months	Adapted plants after six storage months
Soil: Perlite: Coco substrate (1:1:1)	70	60
Soil: Perlite: Coco substrate (2:1:1)	65	40
Soil: Perlite: Sand (1:1:1)	95	70
Soil: Perlite: Sand (2:1:1)	80	50

All plants developed very well after transplanting in the field conditions (Fig. 3d). In vitro propagation is often restricted by high percentage of plant loss when transferred from in vitro to ex vitro conditions. Both factors are of great importance for the success of ex vitro adaptation: availability of well developed roots and controlled reduction of humidity. Well developed root system of the micropropagated plants and the appropriate substrate provides rapid acclimatization at ex vitro conditions. For a successful ex vitro acclimatization of *S. rebaudiana* microplants was reported (Das *et al*., 2011; Sharma and Shahzad, 2011; Razak *et al.,* 2014). Generally, ½ MS medium with 0.1 mg L^{-1} IBA was used during all experiments (in vitro storage, plant recovery and in vitro rooting after storage), except of the media in the propagation stage, which significantly reduces the cost of a plant.

CONCLUSION

An effective protocol for in vitro conservation of *S. rebaudiana* under slow growth conditions was established. Growth reduction was attained by modifying culture medium composition through addition of the osmotic regulator mannitol. A concentration of 3% mannitol in 50% of ionic strength MS was suitable for growth suppression which helped the survival of the plants in a culture up to six months without any subculture under low light intensity, followed by good recovery on fresh ½ MS medium**.** The recovered plants were mass micropropagated, in vitro rooted, and successfully transferred from in vitro to ex vitro conditions. The protocol could be used for medium term in vitro storage of valuable plant individuals and elite genotypes of *S*. *rebaudiana*, which subsequently to be rapidly multiplied for breeding programs or for producing healthy planting material on large scale.

REFERENCES

Ammara, M., Raima, T., Samra, C., Azmat, R., Javed, S. and Khanam, S. 2014. A Review: Role of tissue culture (in-vitro) techniques in the conservation of rare and endangered species. *Pacific Journal of Life Sciences*, **2**(2): 93-103.

Chaeoensub, R., Phansiri, S. 2004. In vitro Conservation of Rose coloured leadwort: Effect of mannitol on Growth of plantlets. *Kasetsart Journal Natural Science*, **38**: 97-102.

Cruz-Cruz, C.A., Gónzalez-Arnao, M.T., Engelman, F. 2013. Biotechnology and conservation of plant biodiversity. *Resources,* **2***:*73-95.

Das, A., Gantait, S., Mandal, N. 2010. Micropropagation of an elite medicinal plant: *Stevia rebaudiana* Bert. *International Journal of Agricultural Research*, **6**(1): 40-48.

Engelmann, F. 2011. Use of biotechnologies for the conservation of plant biodiversity. *In Vitro Cellular & Developmental Biology - Plant***,** **47**(1): 5-16.

Felippe, M. and Lucas, C. 1971. Estudo da viabilidabe dos frutos de *Stevia rebaudiana*. *Hoehnea***,** **1**: 95-105.

Ghauri, G.E., Afridi, M.S., Marwat, G.A., Rahman, I. and Akram, M. 2013. Micropropagation of *Stevia rebaudiana* Bertoni through root explants. *Pakistan Journal of Botany*, **45**(4): 1411-1416.

Hassan, N., Halwagi, El A., Gaber, A., Awady, El M., Khalaf, A. 2007. Slow-growth in vitro conservation of garlic cultivars grow in Egypt: Chemical characterization and molecular evaluation. *Global Journal of Molecular Sciences*, **2**(2): 67-75.

Hassan, N. and Bekheet, S.A. 2008. Mid-term storage and genetic stability of Strawberry tissue cultures. *Research Journal of Agriculture and Biological Sciences*, **4**(5): 505-511.

Holobiuc, I., Blindu, R., Carasan, M., Helepciuc, F., Voichita, C., Negrean, G. 2008. In vitro conservation strategy in *Veronica multifida* spp capsellicarpa (Dubovik) A. Jelen. *Romanian Journal of Biology-Plant Biology*, **53**(2): 71-81.

Hossain, M.A., Shamimkabir, A.M., Jahan, T.A., Hassan, M.N. 2008. Micropropagation of Stevia. *International Journal of Sustainable Crop Production,* **3**(4): 1-9.

Ibrahim, A.I., Nasar, M.I., Mohammed B.R. and Zefzafi M.E. 2008. Plant growth regulators affecting in vitro cultivation of *Stevia rebaudiana*. *Sugar Technology*, **10**(3): 254-259.

Jain, P., Kachhwaha, S., Kothari, S.L. 2009. Improved micropropagation protocol and enhancement in biomass in *Stevia rebaudiana* Bertoni by using high copper levels in the culture medium and chlorophyll content *Scientia Horticulturae*, **119**: 315–319.

Joshi, V. and Kumar, S.J. 2013. Effect of temperature and media supplements on slow growth conservation of medicinal plant *Spilanthes acmella*, *Botanica Serbica*, **37**(2), 155-160.

Kasagana, N.V. and Karumuri, S.S. 2011. Conservation of Medicinal Plants (Past, Present & Future Trends). *J. Pharm. Sci. & Res.* **3**(8), 1378-1386.

Laribi, B., Rouatbi, N., Kouki, K. and Bettaieb, T. 2012. In vitro propagation of *Stevia rebaudiana* Bertoni. A non caloric sweetener and antidiabetic medicinal plant. *International Journal of Medicinal and Aromatic Plants,* **2**(2): 333-339.

Hemant, L., Chandra, S., Wang, Y.H., Sohly M. and Khan A.I. 2014. In vitro Germplasm Conservation of Elite *Stevia rebaudiana* Bertoni, *Acta Horticulture*, **1039**: 303-308.

Mehta, J., Sain, M., Sharma, Dev R., Gehlot, P., Sharma, P. and Dhaker, J. K. 2012. Micropropagation of an Anti diabetic Plant - *Stevia rebaudiana* Bertoni, (Natural Sweetener) in Hadoti Region of South-East Rajasthan, *India Journal of Biological Sciences*, **1**(3): 37-42.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum*, **15**: 473-497.

Negash, A., Krens, F., Schaart, J., Visser, B. 2001. In vitro conservation of enset under slow-growth conditions. *Plant Cell, Tissue and Organ Culture,* **66**: 107-111.

Negri, V., Tosti, N. and Standardi, A. 2000. Slow-growth storage of single node shoots of apple genotypes. *Plant Cell, Tissue and Organ Culture*, **62**: 159–62.

Rao, N.K. 2004. Plant genetic resources: Advancing conservation and use through biotechnology. *African Journal of Biotechnology,* **3**: 136-145.

Razak, U.A., Ong, C.B., Yu, T.S., Lau, L.K. 2014. In vitro micropropagation of *Stevia rebaudiana* Bertoni in Malaysia. *Brazilian Archives of Biology and Technology*, **57**(1): 23-28.

Sharaf, A., Shibli, A., Kasrawi, A. and Baghdadi, H. 2012. Slow-growth preservation of wild shih (*Artemisia herba-alba* Asso.) microshoots from Jordan. Journal of Food, Agriculture and Environment, **10**(2): 1359-1364.

Sharma, S. and Shahzad, A. 2011. High Frequency Clonal Multiplication of *Stevia rebaudiana* Bertoni, Sweetener of the Future, *Journal of Functional and Environmental Botan*y, **1 (**1): 70-76.

Shatnawi, A.M., Shibli, R.A., Abu-Romman, S.M., Al-Mazra'awi, M.S., Ajlouni, ZI., Shatanawi, WA. and Odeh, WH. 2011. Clonal propagation and cryogenic storage of the medicinal plant *Stevia rebaudiana*. *Spanish Journal of Agricultural Research*, **9**(1): 213-220.

Shibli, R.A., Shatnawi, M.A., Subaih, W. and Ajlouni, M.M. 2006. In vitro conservation and cryopreservation of plant genetic resources: A review. *World Journal of Agricultural Sciences*, **2**: 372-382.

Silva, T.L., Scherwinski-Pereira, J.E. 2011. In vitro conservation of *Piper aduncum* and *Piper hispidinervum* under slow-growth conditions. *Pesquisa Agropecuária Brasileira*, **46**(4): 384-389.

Sivaram, L. and Mukundan, U. 2003. In vitro culture studies on *Stevia rebaudiana*, *In Vitro Cellular & Developmental Biology - Plant*, **39**: 520–523.

Srivastava, M., Purshottam, K., Srivastava, K. and Misra, P. 2013. *In vitro* conservation of *Glycyrrhiza glabra* by slow growth culture. *International Journal of Bio-Technology & Research (IJBTR)*, **3**(1): 49-58.

Tadhani, M.B., Jadeja, R.P., Rena, S. 2006. Micropropagation of *Stevia rebaudiana* Bertoni using multiple shoot culture. *Journal of Cell & Tissue Research,* **6**: 545-548.

Thompson, M.R., Douglas, T.J. Obata-Sasamoto, H. and Thorpe, T.A. 1986. Mannitol metabolism in cultured plant cells, *Physiology Plantarum*, **67**: 365-369.

Zayova, E., Stancheva, I., Geneva, M., Petrova, M., Dimitrova, L. 2013. Antioxidant activity of in vitro propagated *Stevia rebaudiana* Bertoni plants of different origins. *Turkish Journal of Biology*, **37:** 106-113.