Global Journal of Biotechnology & Biochemistry 6 (4): 178-182, 2011
ISSN 2078-466X

Micropropagation of *Stevia rebaudiana* Bertoni. A New Sweetening Crop in Egypt

Mohamed R.A. Abd Alhady

Department of Genetic Resources, Tissue Culture Unit,
Ecology and Dry Land Agriculture Division, Desert Research Center, 11753 El-Matarya, Cairo, Egypt

Abstract: The micropropagation of *Stevia rebaudiana* using stem node segment explants obtained from 2 years-old shrubs were achieved. Cultures were established on Murashige and Skoog (MS) as the medium supplemented with different concentrations of 6-benzylaminopurine (BA) at concentration, 0.0, 0.1 and 0.5 mg/l individually or in combination with Kinetin (Kin) at concentration, 0.0, 0.1 and 0.5 mg/l. The highest survival percentage (90%) as well as growth percentage to survival (100%) was obtained on MS medium supplemented with 0.5 mg/L BA + 0.5 mg/l (Kin). The type of cytokinine was the most important factor affecting shoot multiplication. The highest shoot multiplication rate was obtained from single stem node segment cultured on medium supplemented with BA. Increasing BA concentration promoted shoot multiplication. The maximum number of proliferated shoots was obtained on MS medium supplemented with 2.0 mg/l BAP + 0.5 mg/l kin. However, medium supplemented with kin resulted in elongated shoots. Elongated shoots were separated and rooted on MS medium supplemented with indole butyric acid (IBA) or Naphthalene acetic acid (NAA) at concentration, 0.5, 1.0 and 2.0 mg/l. The maximum root induction (100%) was observed on medium supplemented with 1.0 or 2.0 mg/l IBA. IBA showed to be more significant and effective for rooting than NAA in all concentrations used. High survival, percentage (over 75%) was obtained when the plantlets were transferred to greenhouse conditions

Key words: Stevia rebaudiana · Micropropagation · Shoot tip

INTRODUCTION

Stevia rebaudiana is a perennial shrub of the Asteracae family, native to certain regions of South America (Brazil and Paraguay). It is a natural sweetener plant known as sweet weed, sweet leaf, sweet herb on honey leaf, which is estimated to be 300 times sweeter than sugar cane [1]. The leaves of the plant contain glycosides, which have chemical and pharmacological characteristics suitable for use in human diet as a natural calory- free agent. Diterpene glucosides are 100-400 times more sweeter than glucose [2]. The leaves of *S. rebaudiana* are the source of diterpene glycosides, viz. stevioside and rebudioside [3]. Stevioside is regenerated as a valuable natural sweetening agent because of its relatively good taste and chemical stability [4] (Yamazaki and Flores, 1991. Propagation of *S. rebaudiana* is usually done by stem cuttings, which rooting easily but requires high labour inputs and that makes it costly. Poor seed germination is the major factor limiting large - scale cultivation of this plant [5]. Propagation by seeds does not resulting homogenous plant population, leading to a great variability in the important features like sweetening levels and compositions [6]. Tissue culture is the only tool for the rapid mass propagation of *S. rebaudiana.* However, there have been few reports of its *in vitro* growth [7]. The present study was carried out to standardize a suitable protocol for *in vitro* rapid multiplication of *S. rebaudiana* through nodal stem segments and to introduce a new plant to the flora in Egypt.

MATERIALS AND METHODS

Plant Materials: Actively growing shoots of *S. rebaudiana* were collected as source of explants from plants grown in the greenhouse at the Desert Research Center, Cairo, Egypt. Stem nodal segments of about 1.5 cm

Corresponding Author: Mohamed R.A. Abd Alhady, Tissue Culture Unit, Genetic Resources Department, Ecology and Dry Land Agriculture Division, Desert Research Center, 11753 El-matarya, Cairo, Egypt.

in length were isolated from the shoots. The explants were washed in running tap water and then washed again thoroughly by adding a few drops of Tween-20 to remove the superficial dust particles as well as fungal and bacterial spores. They were then surface sterilized with 1.5% sodium hypochlorite solution for 20 min and finally rinsed six times with sterile double distilled water inside the laminar air flow chamber. The explants were then inoculated aseptically into Murashige and Skoog (MS) medium [8] with different concentrations and combinations of growth regulators.

Nutrient Media and Culture Conditions: The basal nutrient medium containing macro and microelements applied throughout this study was MS medium plus 100 mg/l myo-inositol, 30 g/l sucrose, 7 g/l agar and 0.4 mg/l thiamine HCL. The pH value of the nutrient media was adjusted at 5.7 to 5.8 with adding few drops of either 0.1 N NaOH or 0.1 N HCl, prior to the addition of agar. The media were dispensed into 25 x 150 mm tissue culture tubes, each contained 10 ml of culture medium. Sterilization of the medium was achieved by autoclaving the tubes containing media under pressure of 1.1 kg/cm^2 and at 120°C for 20 minutes. One explant was placed in each test tube cultures that were incubated in a growth chamber at 25°C for 16-h/day photoperiod using cool white fluorescent lamps (300 Lux). The explants were maintained by regular subcultures at 4-week intervals on fresh medium with the same composition.

Establishment Stage: Stem node segments were inoculated on MS medium supplemented with different concentrations of 6-benzylamionpurine (BAP- 0.0, 0.1 and 0.5 mg/L) individually or in combination with kinetin (kin- 0.0, 0.1 and 0.5 mg/l).Survival percentage, growth percentage to survival and average shoot length were evaluated after 6 weeks from culture on establishment medium.

Shoot Multiplication: Multiplication experiments were carried out using the most vigorous shoots from the establishment stage. Stem node segments (2-3 cm long) containing two axillary buds were cut and cultured in various media. MS medium containing the same organic compounds that were used in the establishment medium plus BAP (0.0, 0.1 and 0.5 mg/l) and kin. (0.0, 0.1 and 0.5 mg/l) was experimented. Shoot proliferation was determined after six weeks of culture. The following data were recorded number of new shoots formed per explant and average shoot length (cm).

Indication of Rooting and Acclimatization: For rooting, individual shoots 3-5 cm long were excised from the multiplication media and cultured on MS basal medium containing the previously mentioned organic compounds and supplemented with the auxins indol-3-butric acid (IBA) or Naphthalene acetic acid (NAA), each at three concentrations (0.5, 1 and 2 mg/l). The percentage of rooted shoots, number of roots formed per shoot and average root length (cm) were determined after 6 weeks of culture on the rooting medium. Rooted micro shoots were removed from the culture and the roots were washed in sterile distilled water. The plantlets were then transferred to plastic pots containing peat moss and sand (1:1) in the greenhouse (28 ± 2°C, RH 70-80%). The potted plants were irrigated and initially covered with plastic bags, which was gradually eliminated within four weeks time for completing their acclimatization.

Statistical Analysis: At least ten cultures were raised for each treatment. Analysis of variance and the lowest significant difference (L.S.D) according to Snedecor and Cohran [9] were applied for statistical analysis of data. Survival percentage, growth percentage of survival and average shoot length (cm) were determined.

RESULTS AND DISCUSSION

Establishment of *Stevia Rebaudiana in vitro:* Data obtained after six weeks of culturing showed that stem node segments of *S. rebaudiana* could be established at all tested media including the control medium (free from growth regulators). The highest survival percentage (90%), was obtained when the MS medium was supplement with 0.5 mg/l BA + 0.5 mg/l kin followed by MS medium containing 0.5 mg/l BA + 0.1 mg/l kin. which gave 80% survival percentage, compared with the control which gave the lowest survival value (50%). On the other hand, the growth percentage of survival was (100%) on MS medium that were supplemented with 0.5 mg/l BA + 0.5 mg/L kin

Shoot length was significantly affected by the presence of BA or kin (Table 1). BA at concentration 0.5 mg/l reduced shoots length (2.3 cm) and 0.5 mg/l kin without BA was optimum for shoot length (4.2 cm). The present results are in disagreement with that of Sane *et al*. [10] who found that supplementing the medium with different concentrations of BA, stimulated shoot in *Acacia tortilis*. Similar results are obtained by Skolmen and Mapes [11] working on *Acacia koa* and by Dhawan and Bhojwani [12] working on *Leucaena leucocephala,*

Table 1: Establishment of *Stevia rebaudiana* shoot tips using different combinations of BA and Kin after 4 weeks of culture

Treatments (mg\l)	Survival %	Growth of survival %	Average shoot length (cm)
0.0 BA +0.0 Kin	50	60	2.5
0.1 BA +0.0 Kin	60	67	2.4
0.5 BA +0.0 Kin	70	86	2.3
0.0 BA +0.1 Kin	50	80	2.9
0.1 BA +0.1 Kin	60	83	3.1
0.5 BA +0.1 Kin	80	88	2.9
0.0 BA +0.5 Kin	60	83	4.2
0.1 BA +0.5 Kin	70	86	4.0
0.5 BA +0.5 Kin	90	100	4.0

Table 2: Effect of various combinations of cytokinins on *in vitro* shoot multiplication of *Stevia rebaudiana* after six weeks of culture

Treatments (mg\l)	Average no. of shoots/explant	Average shoot length (cm)
0.5 BA +0.5 Kin	18.1	5.2
1.0 BA +0.5 Kin	25.6	4.5
1.5 BA +0.5 Kin	30.8	4.2
2.0 BA +0.5 Kin	36.9	2.7
0.5 BA +1.0 Kin	24	5.9
1.0 BA +1.0 Kin	28.8	5.0
1.5 BA +1.0 Kin	32.2	4.6
2.0 BA +1.0 Kin	36.7	3.2

Fig. 1 Propagation of *Stevia rebaudiana*.
(A) Establishment of *Stevia rebaudiana* (B) Multiplication of shoots.
(C*) In vitro* rooted plantlet after 6 weeks of culture. (D&E) A plantlet acclimatized in greenhouse.

Table 3: Effect of different concentrations of IBA on rooting of *Stevia rebaudiana* after six weeks of culture.

Treatments (mg\l)	Rooted shoot %	Average no. of roots/explant	Average root length (cm)
0.5 IBA	80	5.3	5.4
1.0 IBA	100	7.1	7.3
2.0 IBA	100	8.4	9.0
0.5 NAA	50	2.8	5.8
1.0 NAA	50	4.4	4.9
2.0 NAA	70	6.6	6.0

El Nour *et al*.[13] working on *Balanites aegytpiaca* found that when added kinetin to the medium,it promoted shoot elongation.

Shoot Multiplication: The shoot initials (1.5 cm) obtained from the *in vitro* established cultures were subjected to be multiplied. The shoot numbers per explant and shoot length varied under various cytokinins concentrations and combinations (Table 2). Between the two cytokinins tested, BA was more effective than kin for shoot multiplication. A similar observation was reported for *Bauhinia variegate* [14], *Balanites aegyptiaca* [15] and *Periploca angustifolia* [16]. The highest shoot multiplication occurred in medium supplemented with 2.0 mg/l BA + 0.5 mg/l kin (36.9 shoots per explant), while the lowest shoot multiplication occurred in MS medium supplemented with 0.5 mg/l + 0.5 mg/l kin. Supplemented kin to the medium enhanced the elongation of shoots. Similar observations were found in medicinal plants for example, in *Gxmnema sylvestre* Komalavalli and Rao [17] and Raha and Roy [18] working on *Holarrhena antidysenteria*. Higher levels of BA are required for multiplication shoot, whereas subsequent transfer to media supplemented with low amounts of BA and the additional of kin promoted shoot elongation (5.9 cm).

Root Indication and Plant Acclimatization: About 4-5 cm long shoot tips, harvested from the multiplication medium, were implanted on MS medium containing different concentration of IBA and NAA. Shoots of *Stevia rebaudiana* rooted under all IBA and NAA treatments. The maximum root induction (100%) was formed on MS medium supplemented with 1.0 and 2.0 mg/L IBA, whereas the application of NAA decreased the root induction (Table 3). IBA was the most significant and efficient auxin type for rooting than NAA in all concentrations used. This result is in agreement with that obtained by Abd Al-Hady *et al*. [11] (2010) who mentioned that IBA gave better response for rooting of *Periploca angustifolia* than NAA. According to El Nour *et al*. [13] IBA did not significantly improve the rooting of *Balanites aegyptiaca,* which disagree with our results. The highest root number per shoot (8.4) was recorded in explant exposed to 2.0 mg/l IBA, while the lowest root number per shoot (2.8) was obtained in explants exposed to 0.5 mg/l NAA. With regard to the root length, 2 mg/l IBA induced a significant root length (9 cm). Similar observation has been reported by Al-Meidda *et al*. [19] on *Rhododendron ponticum*.

Plant Acclimatization: Plantlets with well-developed root systems were transferred to plastic pots containing sand and peat moss mixture (1: 1) and covered with translucent plastic bags to ensure high humidity around the plants. The use of this procedure during the acclimatization phase ensured that most of the plantlets transplanted to *ex vitro* conditions continued to grow vigorously. After two months when the plastic bags were completely removed, 75% of the plantlets survived in the greenhouse and showed no sign of water stress. Thereafter, the regenerated plants showed normal growth.

In conclusion, this study describes a protocol for direct shoot regeneration of *Stevia rebaudiana* from stem node segment explants. This protocol provides a successful and rapid technique that can be used for the propagation and *ex situ* conservation of this important plant.

REFERENCES

1. Liu, J. and S.F.Y. Li, 1995. Separation and determination of Stevia sweeteners by capillary electrophoresis and high performance liquid chromatography. J. Liquid Chromatography, 18(9): 1703-1719.
2. Kinghorn, A.D. and D.D. Soejarto, 1985. In: Hikini H, Fransowrth NR, Editors Economical and Medicinal Plant Research, Vol. 1. New York: Academic Press, pp: 1-52.
3. Yoshida, S., 1986. Studies on the production of sweet substances in *Stevia rebaudiana:* I. Simple determination of sweet glucoides in Stevia plant by thin layer chromato-scanner and their accumulation patterns with plant growth. Jap. J. Crop Sci., 55(2): 189-195.
4. Yamazaki, T. and H.E. Flores, 1991. Examination of steviol glucoides production by hairy root and shoot cultures of *Stevia rebaudiana*. J. Natural Products, 54(4): 986- 992.

5. Goettemoeller and Ching, 1999. Seed germination in *Stevia rebaudiana* Perspective on new crops and new uses. J. Janick (ed.) ASHS press. Alexandria, VA. pp: 510-511.
6. Nakamura, S. and Y. Tamura, 1985. Variation in the main glycosiders of *Stevia (Stevia rebaudiana)* Jpn. T. Trop. Agric., 29: 109- 116.
7. Miyagawa, H. and N. Fujioka, 1986. Studies on the tissue cultures of *Stevia rebaudiaana* and its components: II. Induction of shoot primordial. Plant Medica, 4: 321-323.
8. Murashige, T. and F. Skoog, 1962. A revised medium of rapid growth and essayes within tobacco tissue culture. Physiol. Plant. 15: 573-497.
9. Snedecor, G.W. and G.W. Cochran, 1980. Statistical Methods. 6th Ed. Iowa Univ. press Iowa, U.S.A.
10. Sane, D., A. Borgel, J.L. Verdeil and Y.K. Gassama, 2001. Régénération de vitroplants par embryogenése somatique à partir d embryons zygotiques immatures chez une espéce adaptée à la sécheresse: *Acacia torilis* subsp. Raddiana (Savi.) Brenan. Acta Bot. Gall. 147: 257-266.
11. Skolmen, R.G. and M.O. Mapes, 1976. Acacia Koa Gray plantlets from somatic callus tissue. J. Hered., 67: 114-115.
12. Dhawan, V. and S.S. Bhojwani, 1985. *In vitro* vegetative propagation of Leucaena leucocephala (Lam.) de wit. Plant Cell Rep., 4: 315-318.
13. El-Nour, M.E., K. Massimo and B. El-Hassen, 1991. Preliminary study on seed pregermination treatment and vegetative propagation of *Balanites aegyptiaca* (L.) Del. In: Physiology des arbres et Arbustes en zone arides et semi-arides. Groupe d'Etude de l'arbre-Paris, France, pp: 413-415.
14. Ahmed, M.R. and M.H. Bekhit, 2007. *In vitro* propagation of *Balanites aeygptical* (L) Del. an endangered medicinal plant. J. Agric. Sci. Mansoura Univ., 32(4): 2907-2915.
15. Mathur, J. and Mukunthakumar, 1992. Microprogagation of *Bauhinin variegate* and *Parkinsoma aceuleaa* from nodal explants of mature trees. Plant Cell Tiss. Organ. Cult., 28: 119-121
16. Abd-Alhady, M.R., M.M. Abd Alla, G.A. Hegazi and M.F. Gabr, 2010. Rapid propagation of *Periploca angustifolia* Labill. by tissue culture. International J. Plant Developmental Biol., 4(1): 15-18.
17. Komalavalli, N. and M.V. Rao, 2000. *In vitro* microporpagation of *Gymnema sylvestre* - a multipurpose medicinal plant. Plant cell Tiss. Organ. Cult., 61: 97-105.
18. Raha, S. and S.C. Roy, 2001. *In vitro* plant regeneration in *Holarrhena antidysenterica* Wall. though high frequency axillary shoot proliferation. *In vitro* cell. Dev. Biol. Plant. 37: 232-236.
19. Al-Meidda, S.G. and A. Romano, 2005. *In vitro* micropropagation of endangered *Rhododendron ponticum* L. subsp. *Baeticum*. Biodiversity and Conservation, 14: 1059-1069.