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Antioxidant activity of in vitro propagated *Stevia rebaudiana* Bertoni plants of different origins

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Abstract: An efficient in vitro protocol for propagation of *Stevia rebaudiana* Bertoni is described. Multiple shoots were induced in vitro from shoot tip and nodal segments on Murashige and Skoog medium containing 6-benzylaminopurine, zeatin, or thidiazuron alone and in combination with naphthalene acetic acid or indole-3-acetic acid. A high frequency of shoot induction as well as maximum number of shoots per shoot tip explant was observed on Murashige and Skoog medium supplemented with 6-benzylaminopurine (1.0 mg L^{-1}) alone and combined with indole-3-acetic acid (0.1 mg L^{-1}). For root induction, in vitro shoots were transferred to rooting media containing naphthalene acetic acid, indole-3-acetic acid, or indole-3-butyric acid. The highest rooting frequency and the highest number of roots was observed in half-strength Murashige and Skoog medium supplemented with 0.1 mg L^{-1} indole-3-butyric acid. The rooted in vitro plants were successfully acclimatized in a growth chamber and transferred to the field. Leaf extracts of plants propagated in vitro and adapted to field conditions are characterized by high levels of water-soluble antioxidant capacity (expressed as equivalents of ascorbic acid), phenols, and flavonoids, and therefore by high total antioxidant potential, expressed as DPPH radical scavenging activity.

Key words: Acclimatization, micropropagation, nodal segments, shoot tips

1. Introduction

Stevia rebaudiana Bertoni, belonging to the family *Asteraceae*, is a perennial sweet herb. It is a native medicinal plant of Paraguay and is a new alternative source of calorie-free sweetener having no carbohydrates. The leaves of this plant produce diterpene glycosides (stevioside and rebaudiosides). Pure stevioside is 30 times sweeter than sugar (1–4). Recently, food-derived antioxidants, such as vitamins and phenolic phytochemicals, have received growing attention because they are known to function as chemopreventive agents against oxidative damage (5). The dry extract from the leaves also contains flavonoids, alkaloids, water-soluble chlorophylls and xanthophylls, hydroxycinnamic acids (caffeic, chlorogenic, etc.), neutral water-soluble oligosaccharides, free sugars, amino acids, lipids, essential oils, and trace elements (6). Plants constitute an important source of active natural products, which differ widely in terms of structure, biological properties, and ways of propagation. Therefore, it is of great interest to evaluate the nonenzymatic antioxidants and the water-soluble and lipid-soluble antioxidant capacities (expressed as equivalents of ascorbate and α-tocopherol), total phenolic compounds, flavonoids, and free radical scavenging activity of *Stevia rebaudiana* Bertoni propagated in different ways. Although phenolic compounds do not have any nutritional function, they may be important to human health because of their antioxidant potential (7). Therefore, the study of the importance and role of nonnutrient compounds, particularly phenolic acids, flavonoids, and high molecular tannins, as natural antioxidants has greatly increased (8). Natural antioxidants such as α-tocopherol and ascorbic acid are widely used because of their free radical scavenging activity (9). The leaf extract of the stevia plant has been used in the treatment of diabetes (10). It also enhances weight reduction, prevents dental caries, and has antimicrobial properties. It is reported that *S. rebaudiana* Bertoni also contains an antioxidant, steviol (11,12).

This species can be propagated by seed, by vegetative cutting, and by tissue culture. Seed germination is very poor, commonly due to infertile seed (13). Vegetative propagation by stem cutting is limited and requires enough stocks of stem cuttings (14,15). Thus, the development of an efficient alternative method for mass micropropagation of *S. rebaudiana* Bertoni is important for large-scale plant production. A number of protocols for in vitro propagation of this species have been described during recent years (16–23). According to Cenkci et al. (24), both

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conventional and in vitro techniques could be useful for large-scale multiplication and propagation of plant species. There are different factors that influence the process of micropropagation, such as initial explants, genotype, and concentration and combination of growth regulators.

The favorable soil and climatic conditions in Bulgaria also allow the successful growth of *S. rebaudiana* Bertoni, but only as an annual plant because rhizomes cannot survive in low soil temperatures in winter. The seeds show variation among the plants regarding growth and sweetness (25). The necessity of obtaining a large number of identical plants requires the development of an alternative method for propagation.

The objective of this study was to develop an efficient in vitro clonal propagation system for *S. rebaudiana* Bertoni and to evaluate the antioxidant potential of the plants of different origin, both propagated in vitro and adapted to field conditions.

2. Materials and methods

2.1. Initial plant materials

S. rebaudiana Bertoni seeds were obtained from the commercial seed source company Stevia-Paraguay, Paraguay (PY), and from the company Pase Seeds SKU, USA. Plants of the following origins were studied: St PY (Stevia-Paraguay) and St USA (Stevia-United States of America). The seeds were stratified for 1 month at 4 °C. They were carefully washed thoroughly under running tap water. Seeds were then soaked in 70% ethanol for 2 min and agitated in 15% bleach solution (ACE Procter & Gamble Co., USA) containing 5% active chloride for 20 min, and were then washed again 3 times each for 15 min in sterilized distilled water. The disinfected seeds were germinated in Murashige and Skoog (26) basal medium (MS) supplemented with 30 g L^{-1} sucrose, 0.4 mg L^{-1} gibberellic acid (GA_3), and 7 g L^{-1} plant agar (DUCHEFA, the Netherlands). The percentage of germination was determined after 21 days of culture.

2.2. Shoot multiplication

Shoot tip and nodal segments were aseptically excised and cultured on MS media with vitamins containing 0.5 or 1.0 mg L^{-1} 6-benzylaminopurine (BAP), zeatin (Zea), and thidiazuron (TDZ), alone or in combination with 0.1 mg L^{-1} α-naphthalene acetic acid (NAA) or indole-3-acetic acid (IAA) for shoot multiplication (Table 1). The explants were placed in the culture vessels (tubes 140 × 20 mm), with 2 explants per 10 mL of medium. New forming shoots were ready for further subculture 4 weeks after culture initiation. The frequency of shoot formation, number of developed shoots, and length of developed

Table 1. Effect of plant growth regulators on shoot induction of *Stevia rebaudiana* Bertoni (USA origin).

Plant growth regulators	Concentration (mg L^{-1})	Shoot tips		Nodal segments	
		Shoot formation (%)	Shoot number	Shoot formation (%)	Shoot number
BAP	0.5	65	2.6 ± 0.21^{c}	40	1.8 ± 0.17^{def}
	1.0	90	5.4 ± 0.45^{f}	70	3.6 ± 0.29^{i}
Zea	0.5	60	1.7 ± 0.16^{ab}	60	1.4 ± 0.06^{abc}
	1.0	70	3.1 ± 0.38^{d}	65	1.9 ± 0.21^{ef}
TDZ	0.5	40	1.5 ± 0.12^{ab}	30	1.2 ± 0.08^{ab}
	1.0	55	1.8 ± 0.18^{b}	40	1.5 ± 0.14^{bcd}
BAP + NAA	1.0 + 0.1	80	3.6 ± 0.29^{e}	75	2.8 ± 0.26^{h}
BAP + IAA	1.0 + 0.1	95	6.1 ± 0.44^{g}	80	3.7 ± 0.35^{i}
Zea + NAA	1.0 + 0.1	65	1.3 ± 0.11^{a}	50	2.1 ± 0.21^{fj}
Zea + IAA	1.0 + 0.1	55	2.8 ± 0.25^{cd}	65	2.4 ± 0.26^{gh}
TDZ + NAA	1.0 + 0.1	45	1.4 ± 0.12^{ab}	40	1.1 ± 0.05^{a}
TDZ + IAA	1.0 + 0.1	50	1.7 ± 0.15^{ab}	45	1.6 ± 0.12^{cde}
LSD	-	-	0.449	-	0.346

Note: Data are presented as means of 20 individuals per treatment ± standard error. Different letters indicate significant differences assessed by the Fisher LSD test ($P \leq 0.05$) after performing ANOVA multifactor analysis. Shoot formation and shoot number were determined after 4 weeks of cultivation on MS solid media supplemented with various concentrations and combinations of the used plant growth regulators.

shoots were determined. Cultures were subcultured into fresh media once every 4 weeks. Each treatment consisted of 20 replicates and each experiment was repeated 2 times.

2.3. Rooting of plantlets

Individual well-developed shoots were dissected and placed onto the rooting medium (half-strength MS + 20 g L^{-1} sucrose + 0.1 mg L^{-1} indole-3-butyric acid [IBA] + 7 g L^{-1} agar). Results concerning percentage rooting, mean number of roots per plantlet, and root length were reported after 4 weeks of culture. The pH of all media was adjusted to 5.8 with 1 N NaOH or 1 N HCl before autoclaving at 121 °C for 20 min.

2.4. Culture conditions

All in vitro cultures were maintained in a growth chamber at 24 ± 2 °C and a 16-h photoperiod under 40 µmol m^{-2} s^{-1} illumination provided by 40-W cool-white fluorescent lamps during all growth stages of micropropagation. Explants were checked daily during the first weeks for symptoms of contamination and thereafter weekly for signs of growth and development.

2.5. Acclimatization of plantlets and transfer to soil

The plantlets with a well-developed root system were carefully removed from the culture vessels and their roots were washed to remove agar. They were then transferred to small pots (8 cm in diameter) containing a mixture of soil, sand, and perlite in a volume ratio of 1:1:1. The plantlets were maintained in a growth chamber at 25 ± 1 °C and a 16-h photoperiod under 50 µmol m^{-2} s^{-1} illumination provided by cool-white fluorescent lamps. Potted plantlets were covered with a transparent polythene membrane to ensure high humidity (90%). The polythene was opened after 2 weeks. The survival efficiency, defined as the percentage of plantlets that survived the transfer from in vitro to ex vitro growth conditions, was determined 5 weeks after acclimatization. After 2 months of acclimatization, plantlets were transferred to a greenhouse for further growth development. Finally, they were transferred to field conditions.

The leaves of in vitro propagated plantlets were collected from the culture vessels at the rooting stage, and the leaves of the cloned plants grown in the field were collected at the end of September (16 weeks after the planting). The leaves were dried at room temperature.

2.6. Antioxidant activity

Spectrophotometric quantification of water-soluble and lipid-soluble antioxidant capacity (expressed as equivalents of ascorbate and α-tocopherol) was performed through the formation of phosphomolybdenum complex (27). The assay was based on the reduction of Mo(VI) to Mo(V) by the sample analysis and the subsequent formation of a green phosphate/Mo(V) at acidic pH.

Plant dry material (0.5 g) was ground with a mortar and pestle to a fine powder. Next, 3 mL of dH_2O was added and the suspension was homogenized, transferred to tubes, and shaken for 1 h at room temperature for 1 h in the dark. The suspension was filtered and extraction was repeated with 3 mL of dH_2O. The pellet was washed again with 2 mL of dH_2O. For lipid-soluble antioxidant capacity (expressed as α-tocopherol), the procedure was the same but the extraction was carried out with hexane as a solvent. The method has been optimized and characterized with respect to linearity interval, repetitivity and reproducibility, and molar absorption coefficients for the quantitation of water-soluble and lipid-soluble antioxidant capacities, expressed as equivalents of ascorbate and α-tocopherol (27). Absorption coefficients were (3.4 ± 0.1) × 103 M^{-1} cm^{-1} for ascorbic acid and (4.0 ± 0.1) × 103 M^{-1} cm^{-1} for α-tocopherol.

Total antioxidant capacity (free radical scavenging activity) was measured from the bleaching of the purple-colored methanol solution of free stable radical (diphenyl picrylhydrazyl [DPPH•]) inhibition, following the method of Tepe et al. (28). DPPH• radical is a stable radical with a maximum absorption at 517 nm that can readily undergo reduction by an antioxidant. The percentage inhibition of free radical DPPH• (I%) was calculated in the following way:

$$I\% = (A_{blank} - A_{sample} / A_{blank}) \times 100,$$

where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound), and A_{sample} is the absorbance of the test compound, i.e. puncture vine extracts.

For determination of the phenols and flavonoids, dry leaf samples (1 g) were ground and exhaustively extracted with 96% (v/v) methanol. Contents of phenolic compounds were determined spectrophotometrically using Folin-Ciocalteu reagent and calculated as caffeic acid equivalents (29). Flavonoids in plant tissues were measured by the method of Zhishen et al. (30) spectrophotometrically using the standard curve of catechin.

2.7. Statistical analysis

Twenty plants were raised for each treatment and all the experiments were repeated twice. Data were subjected to one-way analysis of variance (ANOVA) for comparison of means, and significant differences were calculated according to Fisher's least significance difference (LSD) test at the 5% level using a statistical software package (StatGraphics Plus, version 5.1 for Windows). Data are reported as means ± standard error.

3. Results and discussion

3.1. Micropropagation of *S. rebaudiana*

The choice of suitable explants and the subsequent decontamination procedure play a major role in successful culture establishment. Surface sterilization with sodium hypochlorite (15%) for 20 min was successful in ensuring

contamination-free seedlings. The seeds were cultured on MS medium containing 0.4 mg L^{-1} GA_3, and they started to germinate 14 days after planting. The germination percentages were 30% and 55% on day 21, respectively, for the seeds of the 2 origins (PY and USA). In our experience, seed germination rate was better after the cool stratification treatment compared with that of nonstratified seeds. They did not germinate in the medium with 0.4 mg L^{-1} GA_3. The seedlings were subcultured on the same medium twice, until the beginning of their stable growth and development. The problem of poor germination of seeds has been reported by many authors (1,15,23). The shoot tips and nodal segments taken from young in vitro plantlets were suitable for the initiation of in vitro culture of *S. rebaudiana* Bertoni. The shoot tip explants possessed a higher potential of multiplication than the nodal segments.

The present study demonstrates the potential for the mass propagation of *S. rebaudiana* Bertoni. The effects of cytokinins BAP, Zea, or TDZ applied alone or in combination with auxins NAA or IAA on the shoot induction were evaluated (Table 1). Shoot formation was observed as a result of plant growth regulator application. The highest number of shoots (an average value of 4.5 shoots per tip explant and an average value of 3.1 shoots per node) was obtained on MS medium supplemented with 1.0 mg L^{-1} BAP. Among all tested concentrations of Zea for shoot formation, the tip explants responded the best at 1 mg L^{-1} (Table 1). The frequency of shoot multiplication was comparatively lower on TDZ-supplemented medium for the 2 types of explants. Among the different cytokinins tested, BAP at the concentration of 1.0 mg L^{-1} exhibited the best effectiveness regarding shoot formation. Shatnawi et al. (23) reported that BAP was the most efficient cytokinin for the axillary shoot initiation and subsequent shoot proliferation. Other authors have also described the superiority of BAP in inducing *S. rebaudiana* Bertoni shoots (17,22,31,32). The values of the propagation frequency and shoot number were higher in the plants of USA origin compared to those of PY origin on all tested MS media supplemented with cytokinins alone (Figure 1).

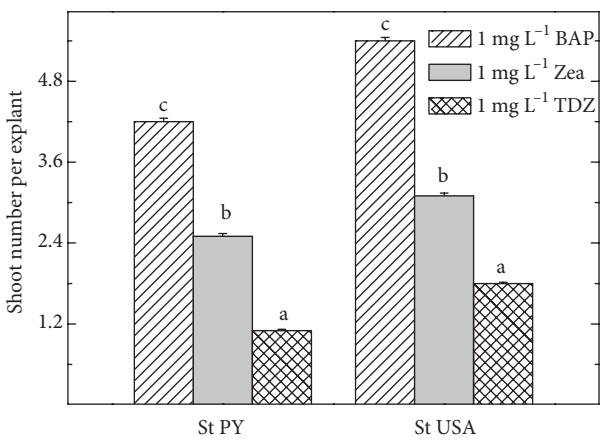

Figure 1. Influence of stevia origin on the number of shoots per explant grown on MS media supplemented with cytokinins. Data are presented as means from 20 individuals ± standard error. Different letters indicate significant differences assessed by the Fisher LSD test ($P \leq 0.05$) after performing ANOVA multifactor analysis.

The type and concentration of plant growth regulators used in the medium may result in the occurrence of a large number of new shoots. Among the different combinations tested in the present study, BAP (1.0 mg L^{-1}) and IAA (0.1 mg L^{-1}) were found to be the most effective for shoot multiplication (Table 1). The highest number of shoots (an average value of 6.1 shoots per tip explant and an average value of 3.7 shoots per node) was obtained on this hormonal combination (Figure 2a). Shoot number per explant was also affected by the nutrient medium supplemented with BAP and NAA (the average number reached 3.6 and 2.8 for the 2 explants, respectively). The other nutrient media examined, containing the combinations Zea/IAA, Zea/NAA, TDZ/IAA, and TDZ/NAA, were not effective for shoot induction. Several authors reported that the combinations of BAP with IAA or NAA in MS medium were suitable for shoot multiplication of *S. rebaudiana* Bertoni, but they used higher concentrations of these growth regulators (20,22). In the present study, the propagation efficiency of tip explants was significantly higher than that of nodal segments, which is consistent with the reports of Hossain et al. (32).

3.2. Rooting and acclimatization of plantlets

The incorporation of auxin in the medium generally promoted plant rooting. Excised shoots (2 cm in length) were cultivated on half-strength MS (½ MS) basal medium with 3 different types of auxins for root development. The rooting responses are summarized in Table 2. In the control medium without auxin, root formation was not observed. The addition of auxin in the medium increased rooting percentage. Root formation started after 12 days of cultivation. Maximum rooting (100%) and mean number of roots per plantlet (4.0 and 3.7, respectively) for St PY and St USA treatments were observed on ½ MS medium supplemented with 0.1 mg L^{-1} IBA (Figure 2b), followed by that observed on ½ MS medium supplemented with 0.5 mg L^{-1} IBA (80% and 90%, respectively). The auxin NAA (0.5 mg L^{-1}) was also effective for root induction (Figure 2c), but a concentration of 1 mg L^{-1} resulted in callus formation at the base of the shoot. The in vitro grown shoots cultured in all media containing IAA gave long roots, which break off easily from the plants during their transfer from in vitro to ex vitro conditions. The largest number of roots was formed on ½ MS medium supplemented with 0.5 mg L^{-1} IAA (Figure 2d).

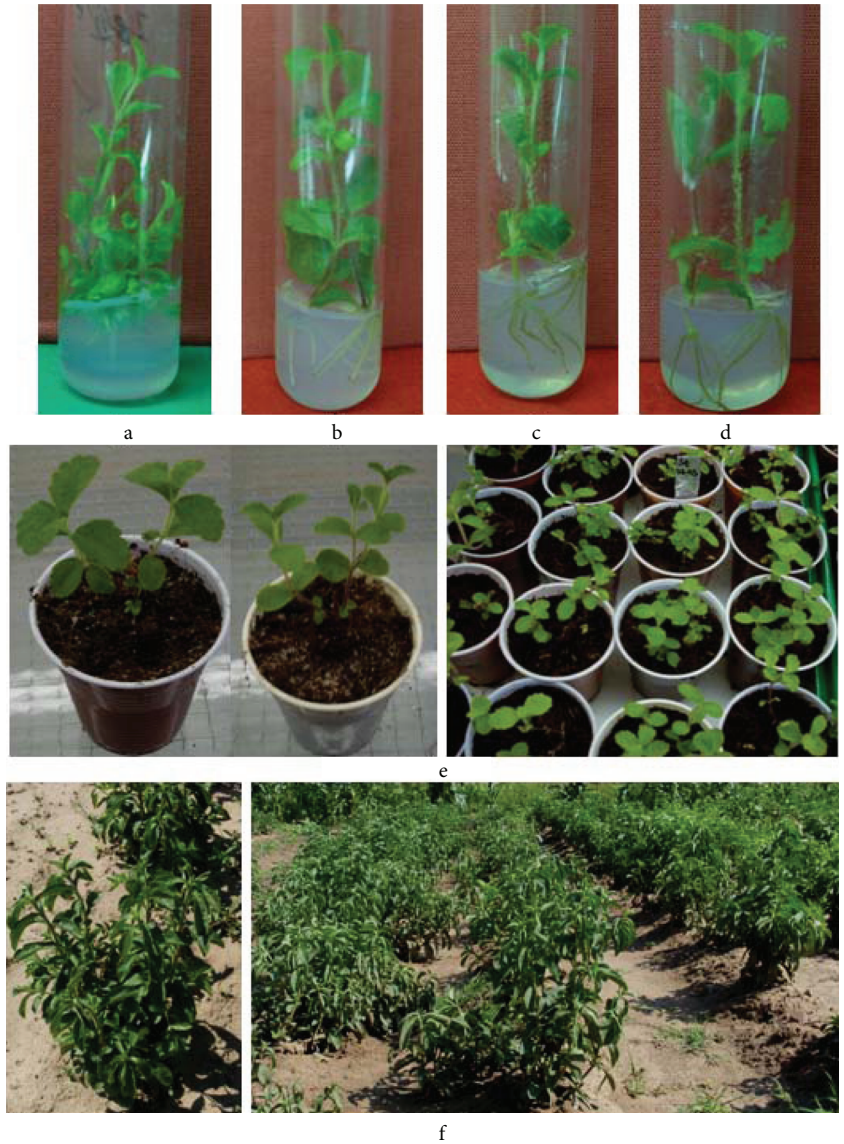

Figure 2. In vitro propagation of *S. rebaudiana*: a) shoot formation from shoot tips on MS + 1 mg L^{-1} BAP + 0.1 mg L^{-1} IAA; b) rooted plantlets on half-strength MS + 0.1 mg L^{-1} IBA; c) rooted plantlets on half-strength MS + 0.5 mg L^{-1} NAA; d) rooted plantlets on half-strength MS + 0.5 mg L^{-1} IAA; e) acclimatized plantlets in soil : sand : perlite; f) stevia plants grown in the field.

The root lengths varied in all ½ MS basal media with NAA, IAA, or IBA applied in different concentrations (Table 2). Observation showed that IBA generally performed better as an auxin for rhizogenesis than NAA or IAA. Debnath (20) and Satpathy and Das (22) obtained the highest rooting using 2.0 mg L^{-1} IBA. On the other hand, Ahmed et al. (19) reported the highest rooting percentage on MS medium with 0.1 mg L^{-1} IAA. In contrast, Hossain et al. (32) observed roots at high frequency on full MS supplemented with 1.5 mg L^{-1} NAA. The shoots were successfully rooted (100%) on MS with 2.0 mg L^{-1} of IBA, IAA, or NAA (23). Uçar Türker et al. (33) reported that IBA is more effective in terms of the number of roots developed, producing a mean of 7.1 roots per shoot at 4.92 µM, whereas IAA was more effective for the frequency of shoots developing roots: 100% of the shoots rooted at 5.71 µM IAA.

The mixture of soil, sand, and perlite (1:1:1) was found to be most appropriate for ex vitro adaptation in comparison with many other tested substrates (data not shown) for this medicinal species (Figure 2e). After acclimatization in the greenhouse, the plants were hardened within a period of 2

Table 2. Effect of the auxins on the root formation of in vitro grown microplants of *Stevia rebaudiana* Bertoni.

Auxins	Concentration (mg L^{-1})	Rooted plants (%)		Root number		Root length, cm	
		St PY	St USA	St PY	St USA	St PY	St USA
Control	0	0	0	0	0	0	0
NAA	0.1	65	70	2.6 ± 0.23^{cd}	2.8 ± 0.32^{b}	1.7 ± 0.15^{cd}	2.1 ± 0.19^{cd}
	0.5	70	80	3.1 ± 0.34^{e}	3.4 ± 0.28^{c}	2.1 ± 0.18^{ef}	2.3 ± 0.23^{d}
	1.0	60	65	2.0 ± 0.19^{a}	2.0 ± 0.14^{a}	1.9 ± 0.21^{de}	2.0 ± 0.21^{bcd}
IAA	0.1	70	75	2.1 ± 0.20^{ab}	2.5 ± 0.22^{ab}	1.5 ± 0.12^{bc}	2.1 ± 0.18^{cd}
	0.5	75	80	3.0 ± 0.18^{de}	3.6 ± 0.34^{cd}	2.6 ± 0.24^{g}	3.5 ± 0.31^{e}
	1.0	65	70	2.4 ± 0.16^{abc}	2.8 ± 0.31^{b}	2.3 ± 0.25^{f}	1.9 ± 0.22^{abc}
IBA	0.1	100	100	3.7 ± 0.35^{f}	4.0 ± 0.42^{d}	1.3 ± 0.11^{ab}	1.6 ± 0.15^{a}
	0.5	80	90	3.0 ± 0.21^{de}	3.6 ± 0.32^{cd}	1.1 ± 0.08^{a}	2.2 ± 0.24^{cd}
	1.0	45	60	2.5 ± 0.27^{bc}	2.8 ± 0.26^{b}	1.5 ± 0.06^{bc}	1.7 ± 0.16^{ab}
LSD				0.424	0.512	0.289	0.369

Note: Data are presented as means of 20 individuals per treatment ± standard error. Different letters indicate significant differences assessed by the Fisher LSD test ($P \leq 0.05$) after performing ANOVA multifactor analysis. Effect of the auxins on the root responses was determined after 4 weeks of cultivation on half-strength MS solid media containing 0, 0.1, or 0.5 mg L^{-1} NAA, IAA, or IBA.

weeks. They were then planted and successfully established in the field (Figure 2f). Therefore, the technique described here provides a promising method for rapid propagation of this economically important medicinal plant species.

3.3. Antioxidant activity

The composition of phenolics and vitamins and antioxidant activities in the extract of *S. rebaudiana* Bertoni are not well known (6). Insufficient data exist regarding antioxidant activity of leaf extracts. Tadhani et al. (9) reported for the first time results about the antioxidant activity of stevia leaves and callus. The protective effects of plant constituents are due to the presence of several components that have distinct mechanisms of action, including low molecular weight compounds such as vitamins, flavonoids, and other phenolic compounds.

The content of water-soluble and lipid-soluble antioxidant capacities (expressed as equivalents of ascorbate and α-tocopherol) and total phenols are higher in the leaves of in vitro multiple plantlets of PY origin in comparison with plantlets of USA origin (Table 3).

High levels of low molecular weight antioxidants led to higher radical scavenging activity in the leaves of plantlets derived from PY seeds. The different origins of *S. rebaudiana* Bertoni (PY and USA) did not significantly influence the level of water-soluble antioxidant capacity, expressed as equivalents of ascorbate, flavonoids, and total antioxidant potential in leaves of the field grown plants. Only lipid-soluble antioxidant capacity expressed as equivalent of α-tocopherol as well as total phenol content were higher in the leaves of USA-origin plantlets. Higher radical scavenging activity in USA-origin plantlets is a result of increased levels of total phenols. Comparatively high levels of water-soluble antioxidant capacity expressed as equivalents of ascorbate in the extracts derived from in vitro propagated and field-adapted plants could be due to the presence of growth regulators such as BAP, NAA, and IBA in the initial growth medium, although such data were not found. Moncaleán et al. (34) suggested that plants "remember" the treatments to which they were exposed during the initial phases of their growth, thus conditioning their future development.

The plants that have higher foliar ascorbate content possess improved tolerance to oxidative stress (35). Vitamin C (ascorbic acid) is required for cardiovascular function, immune cell development, connective tissue, and iron utilization. It is known that total phenols and flavonoids are also involved in plant cell antioxidant defense. According to Benavente-García et al. (36), radical scavenging activity of plant extracts depends on the amount of polyphenolic compounds in the extracts. It has previously been reported that the antioxidant mechanism of flavonoids may also come from the interaction between transition-metal ions and flavonoids to produce complexes that keep the metal ions from participating in free-radical generation (30). The beneficial effects of certain phenols or their potential antioxidant properties, especially when

Table 3. Antioxidant potential in leaves of in vitro multiple and field-grown plants of *Stevia rebaudiana* Bertoni.

Treatments	Water-soluble antioxidant capacity expressed as equivalents of ascorbic acid (μM g DW^{-1})	Lipid-soluble antioxidant capacity expressed as equivalents of α-tocopherol (μM g DW^{-1})	DPPH (%)	Phenols (mg g DW^{-1})	Flavonoids (mg g DW^{-1})
In vitro St PY	61.59[b]	2.25[b]	88.78[b]	8.912[b]	8.01[a]
In vitro St USA	32.94[a]	1.55[a]	70.57[a]	7.44[a]	8.44[a]
LSD	5.60	0.22	9.09	0.92	0.93
Field St PY	262.24[a]	0.68[a]	95.45[a]	14.65[b]	13.04[a]
Field St USA	276.00[a]	0.85[b]	92.03[a]	10.51[a]	13.83[a]
LSD	30.53	0.08	10.63	1.45	1.52

Note: Data are presented as means of 4 replications. Different letters indicate significant differences assessed by the Fisher LSD test ($P \leq 0.05$) after performing ANOVA multifactor analysis.

these compounds are present in large quantities in foods, resulted in supplementation of natural antioxidants through an effective, balanced diet, which is of great importance to consumers. Shukla et al. (37) reported that a significant and linear relationship was found between antioxidant activity and phenolic content in stevia leaf extracts, indicating that phenolic compounds could be major contributors to antioxidant activity. Contents of flavonoids and other phenolic substances have been suggested to play a preventive role in the development of cancer and heart disease (38).

There are many different antioxidants present in plants and it is very difficult to measure each antioxidant component separately. Therefore, several methods have been developed to evaluate the total antioxidant activity of fruits or other plants and animal tissues. DPPH radicals are widely used in the model system to investigate the scavenging activities of several natural compounds. Stevia leaves and callus have strong antioxidant activity and may be rich sources of antioxidants (5,9). Some authors have demonstrated that the ethanolic extract of *S. rebaudiana* leaves contained high levels of total phenolic compounds and were capable of inhibiting and quenching free radicals to terminate the radical chain reaction, acting as reducing agents (37). The ethanolic extract of *S. rebaudiana* Bertoni leaves showed strong antioxidant activity by inhibiting DPPH, hydroxyl radical, nitric oxide, superoxide anion scavenging, and hydrogen peroxide scavenging activities when compared with standard ascorbic acid.

4. Conclusion

An efficient method for micropropagation of *S. rebaudiana* Bertoni using MS solid medium formulations with plant growth regulators was developed. The best nutrient medium for shoot multiplication was MS supplemented with 1 mg L^{-1} BAP and 0.1 mg L^{-1} IAA. Half strength MS medium containing 0.1 mg L^{-1} IBA showed the best results for root formation of plantlets. The protocol obtained possesses potential for large-scale micropropagation and application in plant breeding research programs. It can be concluded that in vitro plant multiplication is a better technique for plant propagation than ordinary plant germination from seeds. Plants propagated in vitro and adapted to field conditions possess higher water-soluble antioxidant capacity, expressed as equivalents of ascorbic acid, and accumulate more antioxidant metabolites such as total phenols and flavonoids, therefore showing higher free radical scavenging activity. Therefore, it can be concluded that the leaf extracts taken from micropropagated and field-adapted plants can be used as a rich source of natural antioxidants.

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