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Rapid *in Vitro* Propagation and Enhanced Stevioside Accumulation in *Stevia rebaudiana* Bert

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A very rapid and efficient regeneration method has been established using mature explants of *Stevia rebaudiana* Bert. Adventitious shoots were induced from nodal explants of field-grown plants on four basal media supplemented with various combinations of auxins and cytokinins. The best performance (23.4 ± 2.1 shoots per explant) was obtained on a Murashige and Skoog (MS) medium containing 2 mg L^{-1} IAA and 0.5 mg L^{-1} kinetin. Roots were then produced when these *in vitro*-regenerated shoots were transferred to an MS medium supplemented with 3% sucrose and 2 mg L^{-1} IBA. When acclimatized to soil, the rooted plants had a 98.4% survival rate. Following transplantation in the field, stevioside contents were similar between the regenerated plants (10.68 mg g^{-1} dry weight) and the mother plants (12.01 mg g^{-1} dw).

Keywords: nodal segments, rooting, shoot multiplication, *Stevia rebaudiana*, stevioside

Stevia rebaudiana (Bert.) is an important sugar substitute. This member of the Compositae family was originated in the highland regions of northeastern Paraguay (23° to 24° S). Plants are now being cultivated in China, Taiwan, Thailand, Korea, Brazil, and Malaysia. Its leaves contain a large amount of the glycoside stevioside (Geuns, 2003), which is formed by three molecules of glucose and one molecule of steviol, a diterpenic carboxylic alcohol. These *Stevia* extracts have long been used in southern Africa to treat diabetes. Their ingestion causes a slight suppression of plasma glucose levels and significantly increased glucose tolerance in normal adult humans (Curi et al., 1986). Intravenous administration of stevioside also results in a clinically significant hypotensive effect in spontaneously hypertensive rats, without adversely affecting their heart rates or serum catecholamine levels (Chan et al., 1998).

Because seed germination rates are very low from *S. rebaudiana*, this approach to propagation does not allow for the production of a homogeneous population, thus creating great variability in important features such as sweetening levels and composition. Furthermore, success in its vegetative propagation is also limited by the low number of individuals that can be obtained simultaneously from a single plant. Therefore, tissue culture techniques hold great promise for micropropagation, conservation, and enhancement of the natural levels of *in vitro* production of valuable compounds (Tamura et al., 1984; Ferreira and Handro, 1987; Swanson et al., 1992; Akita et al., 1994; Bondarev et al., 2003; Chan et al., 2005). The objective of the present investigation was to develop a system for rapid mass micropropagation via nodal segment culture and, subsequently, to establish plantlets in the field. Stevioside contents also were measured for *in vitro*-regenerated plantlets.

MATERIALS AND METHODS

Stock Plants and Disinfection

Young twigs of *S. rebaudiana* (Bert.) were collected in May 2004 from the Herbal Garden at Naju city, Chonnam, Korea. They were surface-sterilized in 50% (v/v) ethanol for 5 min and 0.1% (v/v) sodium hypochlorite solution for 10 min before being washed three times in sterile distilled water. All subsequent operations were performed under aseptic conditions.

Culture Media Preparation and Culturing Conditions

The culture media used for these initiation and multiplication experiments included MS (Murashige and Skoog, 1962), B5 (Gamberg et al., 1968), WPM (Lloyd and McCown, 1980), and SH (Schenk and Hilderbrandt, 1972). Each was supplemented with 30 mg L^{-1} sucrose and 0.3% (w/v) phytagel (Sigma-Aldrich, USA). After the pH was adjusted to 5.8, the media were autoclaved and dispensed into 87 × 25 mm disposable Petri dishes, under aseptic conditions. Five explants were cultured per dish. For the *in vitro* rooting phase, 15 mL of a particular medium was placed in a 20 × 200 mm test tube, which was then covered with aluminum foil and autoclaved. All cultures were incubated at 26 ± 1°C under a 16-h photoperiod, with a light intensity of 50 μmol m^{-2} s^{-1} provided by cool-white fluorescent tubes.

In Vitro Shoot Proliferation

For adventitious shoot initiation, nodal explants were removed from the stock plants and inoculated on one of four media types: MS, WPM, B5, or SH. Five nodal segments per Petri dish were cultured on basal media supplemented with various concentrations and combinations of cytokinins (BA or kinetin) and auxins (IAA, NAA, or 2,4-D). The number of shoots per explant was scored after six weeks of culture; each experiment was replicated five times.

Transfer of Rooted Plants to Soil

The rooted plantlets were removed from their culture vessels, washed, and transplanted into plastic pots containing a 1:3 potting mixture of perlite and vermiculite. After three weeks of acclimatization in a mist tunnel without additional lighting, they were transferred to the field to monitor their performance.

Analysis of Stevioside

The extraction and analysis of stevioside were carried out

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according to the method of Bovanova et al. (1998). For each sample, 1 g of tissue was powdered and extracted with 25 mL of boiling deionized water, with occasional shaking. The extracts were then filtered, pooled, and their volume adjusted to 100 mL with deionized water. A portion of this extract was diluted ten times and cleaned via solid-phase extraction. Stevioside contents were determined by HPLC (Waters, USA) at ambient temperature. Conditions included mobile phase, methanol:deionized water (88:32); flow rate of 0.9 mL min^{-1}; and UV detection at 210 nm. Stevioside was quantified in comparison with an authentic sample (Sigma-Aldrich, USA).

RESULTS AND DISCUSSION

In vitro propagation of adventitious shoots is regulated by several important culturing conditions, including the composition of plant growth regulators in the media, the concentration of the carbon source, and the strength of the macronutrients. Although micropropagation has previously been done with *S. rebaudiana* (Tamura et al., 1984; Ferreira and Handro, 1988; Akita et al., 1994), few shoots have been regenerated. Here, various concentrations and combinations of cytokinins and auxins were tested in different types of media. Adventitious shoot formation was most successful when the media were supplemented with IAA (1.0 to 3.0 mg L^{-1}) in combination with either kinetin (0.5 to 1.0 mg L^{-1}) or BA (0.5 to 1.0 mg L^{-1}); explants placed on PGR-free media did not produce any new shoots. The highest regeneration frequency (99.3%) and the maximum number

Table 1. Effects of plant growth regulators on adventitious shoot formation from nodal segments of *S. rebaudiana*.

PGR (mg L^{-1})				Shoot regeneration (%)	Shoot number (Mean ± SE)
IAA	1.0	Kinetin	0.0	43.3[R]	3.5 ± 2.1
			0.5	82.3[C]	13.2 ± 3.4
			1.0	53.9	5.2 ± 3.1
	2.0	Kinetin	0.0	57.1[R]	4.7 ± 1.3
			0.5	99.3	23.4 ± 2.1
			1.0	69.0[C]	13.6 ± 2.3
	3.0	Kinetin	0.0	46.1[R]	3.2 ± 1.1
			0.5	47.3[R]	5.1 ± 3.2
			1.0	38.9[C]	4.8 ± 1.5
IAA	1.0	BA	0.5	76.8	8.5 ± 1.5
			1.0	44.5	4.5 ± 2.3
	2.0	BA	0.5	69.3	10.2 ± 2.3
			1.0	39.2	3.3 ± 2.1
	3.0	BA	0.5	55.4[C]	6.4 ± 1.3
			1.0	36.7	4.5 ± 1.3

Data (mean ± SE) from five independent experiments, each with five nodal explants, after 6 weeks in culture. R, with rooting; C, callusing.

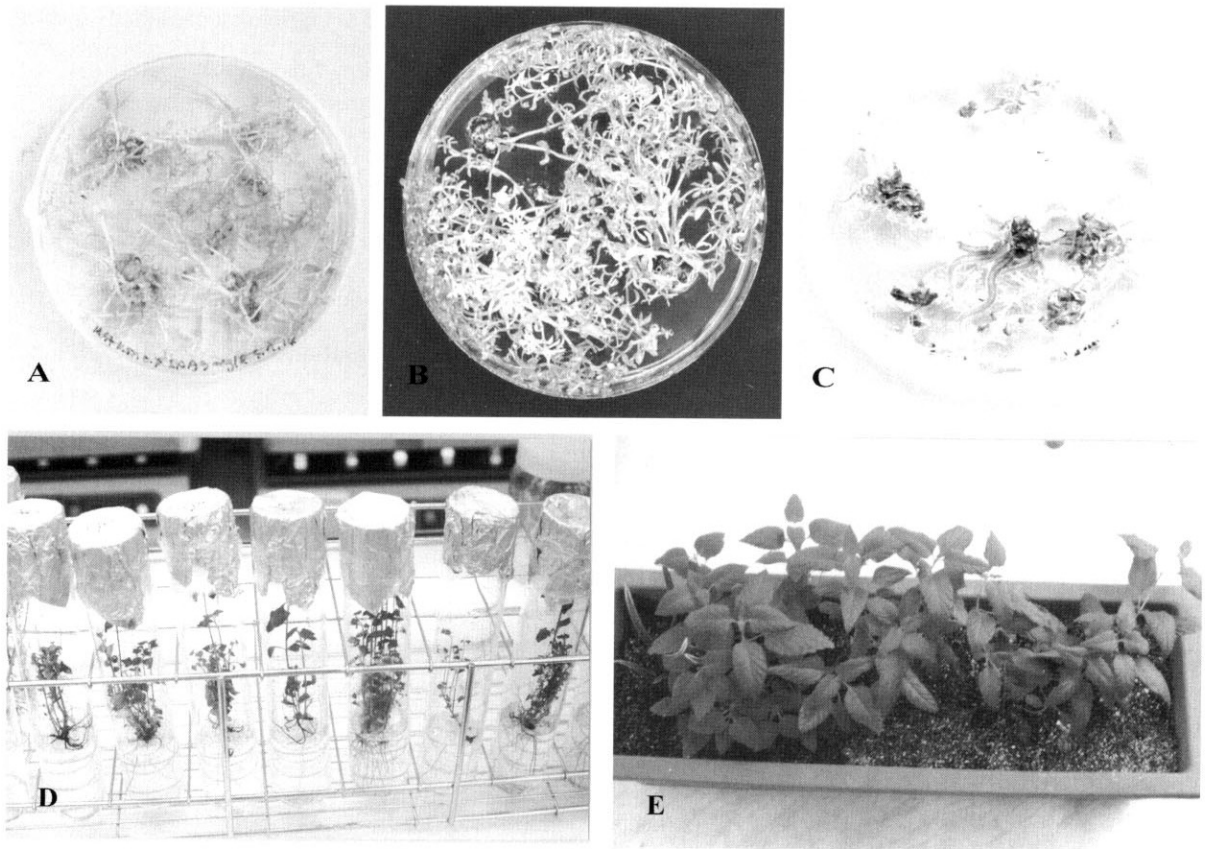

Figure 1. Explant culturing of *S. rebaudiana*. (**A, B**) Shoot regeneration and proliferation on nodal segments 4-6 weeks after culture on MS medium supplemented with 0.5 mg L^{-1} kinetin plus 2 mg L^{-1} IAA, (**C**) Adventitious root formation on MS medium containing 1 mg L^{-1} IBA, (**D**) Rooted plantlet grown on PGR-free MS medium, (**E**) Plants at 6 weeks after transfer to soil, under greenhouse conditions.

Table 2. Effects of basal medium type on adventitious shoot formation from nodal segments of *S. rebaudiana*.

Medium	Shoot regeneration (%)	Shoot number (Mean ± SE)
MS	99.3	23.4 ± 2.1
SH	68.3	7.5 ± 2.3
WPM	47.1	4.3 ± 2.3
B5	63.1	6.5 ± 2.0

Data (mean ± SE) from five independent experiments, each with five nodal explants, after 6 weeks in culture.

of shoots per nodal explant (23.4 ± 2.1) were obtained after 6 weeks of culture on a medium containing 0.5 mg L^{-1} kinetin and 2.0 mg L^{-1} IAA (Table 1; Fig. 1A). This combination was superior in promoting shoot multiplication and growth, with the least amount of callusing at the bases of the explants. Similar results have been reported for witloof chicory (Rehman et al., 2003), *Lepidium sativum* (Pande et al., 2002), *Ammi majus* (Purohit et al., 1995; Pande et al., 2002), and Brassica sp. (Evans et al., 1981). In contrast, the addition of 2,4-D or NAA in the presence of BA induced callus formation while decreasing the number of shoots formed. The use of IAA alone stimulated adventitious root formation (Fig. 1A).

To determine the best type of basal medium for culturing *S. rebaudiana*, mature nodal segments were placed on four commonly used mixtures: MS, WPM, B5, and SH. For each trial, a carbon source (3% sucrose) and plant growth regulators (0.5 mg L^{-1} kinetin and 2.0 mg L^{-1} IAA) were added. Among the four, the MS medium promoted a generally higher rate of regeneration (Table 2), with multiple shoots developing from an outgrowth of axillary branching that resulted in a bushy appearance (Fig. 1B). Sivaram and Mukundan (2003) also have used an MS medium supplemented with 8.87 μM BA and 5.71 μM IAA for the proliferation and differentiation of *S. rebaudiana* shoots, and have found that, in testing various concentrations of sucrose (1 to 5%), a level of 3% is best, with adventitious shoot formation being decreased at lower concentrations.

Rooting and transplantation of plantlets to the field is the most important but difficult task in the micropropagation process (Murashige, 1974). In the current study, the auxin source and concentration significantly affected the success of adventitious root formation. When *in vitro*-produced shoot cuttings (2.0 to 2.5 cm long) were transferred to an MS medium containing 1.0 mg L^{-1} IBA, the rooting response was 100%, and initiation occurred in all treatments within a week of sub-culturing (Fig. 1C). The number of roots per explant was highest (average of 13.2) on 1.0 mg L^{-1} IBA and lowest (2.3) on the PGR-free control medium (Table 3; Fig. 1D). Smith (1983) has reported a success rate as high as 90% by including 0.5 mg L^{-1} IBA in the root-induction medium. Likewise, Trautmann and Visser (1990) have demonstrated rooting rates of up to 90% from 0.3 mg L^{-1} IBA, with root lengths decreasing as the IBA concentration increases. In the case of *S. rebaudiana*, a higher IAA concentration in the media promoted callus proliferation from the bottoms of the shoots that not only arrested their growth but also promoted their deterioration.

Before being transferred to the field, all the *in vitro* plantlets were acclimatized by placing them in plastic pots for 3 to 4 weeks and then shading them during an intermediate stage. Following this pre-treatment, all the transplanted plantlets survived in the field (Fig. 1E), and no significant morphological differences were observed between these acclimatized plants and the field-grown mother plants. Such a transfer of rooted plantlets from *in vitro* culturing conditions to the external environment must be conducted carefully because their leaves lack epicuticular wax and cannot effectively control their stomatal functioning, which can enhance water loss when these plantlets are placed in soil (George and Sherrington, 1984). Sivaram and Mukundan (2003) have reported a 70% survival rate for rooted shoots of *S. rebaudiana* following transplantation. In this present study, the acclimatization and hardening process resulted in 98.4% survival under field conditions.

Table 3. Effects of auxin on *in vitro* rooting of shoots from *S. rebaudiana*.

Auxin (mg L^{-1})		Rooting (%)	Root number (Mean ± SE)
PGR- free		38.4	2.3 ± 2.4
IAA	1.0	68.3	7.8 ± 2.0
	2.0	44.3	4.4 ± 2.0
	3.0	59.2	3.5 ± 2.3
NAA	1.0	33.3	4.2 ± 1.4
	2.0	54.8	5.8 ± 2.0
	3.0	22.5	2.2 ± 2.0
IBA	1.0	100	13.2 ± 1.3
	2.0	78.6	7.5 ± 2.3
	3.0	46.4	3.3 ± 2.1

Data (mean ± SE) from five independent experiments, each with five nodal explants, after 6 weeks in culture.

Table 4. Stevioside contents in cultured tissues versus field-grown plants of *S. rebaudiana*.

Sample		Stevioside content (mg g^{-1} dry weight)
In vitro		
	nodal explant	0.63
	callus	0.14
	Regenerated plant	10.68
In vivo		
	leaves	12.01
	stem	1.42

One of the advantages of plant tissue cultures is that the greater availability of biomass provides researchers with an opportunity to assay the yield of secondary metabolites at specific stages of growth and differentiation (Srivastava et al., 1993; Datta and Srivastava, 1997; Pande et al., 2000). Here, the amounts of stevioside in the leaves, stems, and calli were quantified by HPLC, and its accumulation was compared between these regenerated plantlets and the field-derived plants (Table 4). Nodal cultures of *S. rebaudiana* produced trace amounts of stevioside whereas the regenerants synthesized this glycoside at a rate of 10.69 mg g^{-1} dry weight. Nevertheless, when those plantlets were then transferred to soil and grown in the pots and field, they showed stevioside contents similar to those measured in the mother plants.

In conclusion, the present investigation has demonstrated that, by optimizing the levels of plant growth regulators and the choice of culture media, producers can achieve rapid and large-scale propagation of *S. rebaudiana*, making this a useful technique for the commercial production of stevioside in a suitable bioreactor system.

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