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RESEARCH ARTICLE

In vitro propagation of *Stevia rebaudiana* (Bert.) - A non caloric sweetener and antidiabetic medicinal plant

Bochra LARIBI*, Nadia ROUATBI, Karima KOUKI, Taoufik BETTAIEB

Département d'Agronomie et de Biotechnologies Végétales, Institut National Agronomique de Tunisie. 43, Av. Charles Nicolle-1082, Tunis, Tunisie

Corresponding author, Tel: (+216) 71 28 71 10, Fax: (+216) 71 79 93 91

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Abstract: This paper presents an efficient protocol for rapid *in vitro* propagation of *Stevia rebaudiana* (Bert.) by nodal explants collected from mature plants placed on MS medium containing 1.0 mg l^{-1} 6-benzylamino purine (BAP) or indole-3-butyric acid (IBA) either alone or in combination (1 mg l^{-1} BAP with 0.5 mg l^{-1} IBA) for shoot bud initiation. In order to induce multiple shoots, *in vitro* derived shoot buds from nodal explants were cultured on MS medium supplemented with different concentrations of IBA or indole-3-acetic acid (IAA; 0, 0.25, 0.5 mg l^{-1}) in combination with BAP (1 mg l^{-1}). Shoot buds were also placed on MS medium fortified only with IBA (0.5 mg l^{-1}). Elongated shoots (3 cm) were transferred onto the rooting medium containing MS salts, vitamins and different concentrations of IBA or IAA (0, 0.25, 0.5 mg l^{-1}) used individually for root induction. Results showed that the highest percent response (92.10%) was obtained on MS medium supplemented with BAP (1 mg l^{-1}) and IBA (0.5 mg l^{-1}) and hence this combination was found to be the most effective for shoot bud initiation. In addition, BAP (1 mg l^{-1}) and IAA (0.25 mg l^{-1}) combination was superior for multiple shoot bud induction (4.25 shoots). However, when shoot buds were placed on MS medium fortified only with 0.5 mg l^{-1} IBA, few multiple shoots (1.37 shoots/explant) were induced. In fact, shoots produced roots within two weeks of culture; the highest percent (97%) of root induction and the maximum number of roots (8.90 roots/shoot) as well as the root length (1.70 cm) were observed on MS medium fortified with IAA (0.50 mg l^{-1}). Rooted plantlets were successfully acclimatized in pots containing peat. Thus, this study provides an efficient and reproducible *in vitro* propagation protocol for rapid multiplication of *Stevia rebaudiana* and hence could be helpful to establish and cultivate this medicinal species as a promising newly introduced non caloric sweetener and antidiabetic medicinal plant in Tunisia.

Keywords: *in vitro* propagation; MS medium; nodal explant; plant growth regulators; *Stevia rebaudiana* Bert.

Abbreviations: BAP: 6-benzylaminopurine; IAA: indole-3-acetic acid; IBA: indole-3-butyric acid; MS: Murashige and Skoog.

Introduction

Research on medicinal plants has considerably increased in recent years and is nowadays oriented towards discovering new sources beneficial to human health. Naturally low calorie sweeteners isolated from medicinal plants are gaining a great interest in their use as dietary sucrose substitutes in food and pharmaceutical products. *Stevia rebaudiana* (Bert.) is a perennial sweet herb belongs to Asteraceae family which has great potential as a crop for the production of a high-potency natural sweetener (Savita et al. 2004; Lemus-Mandaca et al. 2012). *Stevia* originates from certain regions of South America, e.g. Paraguay and Brazil but is now widely cultivated in several Asian countries, in some European ones and also all over South America (Brandle et al. 1998). Owing to its proximate composition and its content of health-promoting constituents, *Stevia* is also a suitable raw material for the extraction and production of functional food ingredients. It is a good source of carbohydrates, protein, crude fibre, minerals, as well as dispensable and indispensable amino acids which are valuable for human nutrition. Leaves of *Stevia* contain diterpene glycosides that are low calorie sweeteners, with stevioside being the most abundant, followed by rebaudioside A (Madan et al. 2010; Lemus-Mandaca et al. 2012; Puri et al. 2012).

*Corresponding author: (E-mail) bochra_laribi<A.T.>yahoo.fr
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Thus, the medicinal properties and the commercial value of *Stevia* lead to a high worldwide demand of this non caloric sweetener plant. However, the lower seed germination percentage and lower success of vegetative propagation by stem cuttings were the major limiting factors for large-scale cultivation (Carneiro et al. 1997; Debnath et al. 2006; Taware et al. 2010). Consequently, micropropagation which can provide genetically uniform plants in large numbers appears as an alternative technique for rapid multiplication of *Stevia* plants (Sairkar et al. 2009). In such context, the *in vitro* clonal propagation of *Stevia* was carried by using leaf (Das et al. 2006; Ali et al. 2010; Preethi et al. 2011a,b), nodal and inter-nodal segment (Uddin et al. 2006; Ahmed et al. 2007; Verma et al. 2008; Sairkar et al. 2009; Thiyagarajan and Venkatachalam 2012) and shoot tip explants (Anbazhagan et al. 2010; Giridhar et al. 2010; Das et al. 2011).

Furthermore, many researches on the chemical and biological activities of *Stevia* have been done in recent years and the commercial cultivation has started in several countries (Madan et al. 2010). Nevertheless, no work has been undertaken concerning the *in vitro* propagation or cultivation of *Stevia* in Tunisia. Thus, the National Agronomic Institute of Tunisia (Institut National Agronomique de Tunisie) has recently introduced this sweetener plant for experimental purpose. Accordingly, the present study was undertaken to develop an efficient protocol for rapid *in vitro* propagation of *Stevia rebaudiana* and making thereby a contribution in enhancing its importance as a promising newly introduced non caloric sweetener and antidiabetic medicinal plant in Tunisia.

Materials and methods

Plant material

Nodal explants were collected from 2 months old plants of *Stevia rebaudiana* Bert. These lasts were grown and maintained in the greenhouse, Department of Agronomy and Vegetal Biotechnology, National Agronomic Institute of Tunisia.

Surface disinfection and sterilization

After excision, for surface sterilization, nodal explants were primarily rinsed in running tap water. Further sterilization was carried out in the laminar airflow chamber by using 0.1% (w/v) $HgCl_2$ for 2 min. The explants were then rinsed three times with sterile distilled water and surface sterilized with 10% (w/v) sodium hypochlorite for 2 min followed by rinsing them for 5 min with sterile distilled water. Sterilized nodal explants were used for *in vitro* studies as described below.

Culture media and growth conditions

The culture medium consisted of Murashige and Skoog's (1962) medium (MS) salts and vitamins, and 3% (w/v) sucrose. The medium was gelled with 0.6% (w/v) agar (Sigma) and the pH was adjusted to 5.8 with 0.1 N NaOH or HCl before autoclaving at 120°C for 20 min under a pressure of 1.1 kg cm^{-2}. The cultures were incubated at 23 ± 1°C under 16/8h (light/dark cycle) photoperiod and irradiance (36 µmol m^{-2} s^{-1}) provided by cool-white fluorescent lamps.

Shoot bud initiation

For shoot bud initiation, surface sterilized nodal explants were cultured on MS medium supplemented with 1 mg l^{-1} BAP or 1 mg l^{-1} IBA either alone or in combination (1 mg l^{-1} BAP with 0.5 mg l^{-1} IBA). The MS medium without adding of growth regulators was served as control. After two weeks of culture, percent response was determined and direct shoot bud initiation from nodal explants was noticed.

Multiple shoot bud induction

In order to achieve multiple shoot bud regeneration, the synergistic effect of auxin-cytokinin was evaluated. So, nodal derived *in vitro* regenerated shoot buds as explant source were cultured on MS medium supplemented with different concentrations of IBA or IAA (0, 0.25, 0.5 mg l^{-1}) in combination with 1 mg l^{-1} BAP. The total number of multiple shoots regenerated and the shoot length were recorded.

In vitro rooting of elongated shoots and acclimatization

For root induction, elongated shoots (3 cm) were transferred onto the rooting medium containing MS salts, vitamins and different concentrations of IBA or IAA (0, 0.25, 0.5 mg l^{-1}) used individually. The MS medium without adding of growth regulators was served as control. Data were recorded in terms of percentage of rooting, number and length of roots/shoots after three weeks of culture. The rooted plantlets were transferred to plastic pots filled with peat and covered with polythene bags to ensure high humidity. They were grown under confined conditions before their transfer into the soil under greenhouse.

Statistical analysis

Experiments were conducted as a completely randomized block design. Twenty-four explants were used per treatment in triplicates. Data were subjected to statistical analysis using the program package SAS (SAS 1999). The one-way analysis of variance (ANOVA) followed by Duncan's multiple range test at the significance level of 5% was used to compare means.

Results and discussion

Shoot bud initiation

In the present study, nodal explants from mature *Stevia rebaudiana* plants were placed on MS medium supplemented with 1.0 mg l^{-1} BAP or IBA either alone or in combination (1 mg l^{-1}BAP with 0.5 mg l^{-1} IBA) for shoot bud initiation (Figure 1A).

Table 1: Effect of different concentrations (mg l^{-1}) of BAP and IBA on shoot bud induction from nodal explants of *Stevia rebaudiana* (Bert.).

Growth regulators (mg l^{-1})		Percent response	No. of shoot /explant
BAP	IBA		
-	-	49.50 c	0.68 b
1.0	-	67.52 b	1.10 ab
-	1.0	57.89 c	1.28 ab
1.0	0.5	92.10 a	1.89 a

Mean values within the column followed by the same letter in superscript are not significantly different at $P<0.5\%$.

As shown in table 1, the lower percent response (49.50%) as well as the lower number of shoots induced per explants (0.68 shoots/explants) was observed on the control induction MS medium (Figure 1B). Our results indicate that shoot bud initiation of *Stevia rebaudiana* do not responds well when the nodal explants were cultured on growth regulators-free MS medium. Das et al. (2011) recorded 57.85% percent nodal explants of *Stevia rebaudiana* in MS medium without adding growth regulators.

When MS medium was supplemented with 1 mg l^{-1} IBA or BAP the response was 67.52 and 57.89% respectively; thereafter, a minimum of one shoot per explants was produced (Table 1). The present result are lower than those of Thiyagarajan and Venkatachalam (2012) and Ali et al. (2010) who reported 90 and 85.7%, respectively. They also suggested that the MS basal medium with 1.0 mg l^{-1} BAP was the best one for shoot formation from nodal explants of *Stevia rebaudiana*, which is not in consistent with present findings. Besides, the highest percent response (92.10%) was noticed on MS medium containing 1.0 mg l^{-1} BAP and 0.5 mg l^{-1} IBA, and thereafter the nodal explants produced approximately two shoots within two weeks (Table 1). Moreover, the synergistic effect of BAP and IAA at lower concentration was reported to be optimum for regenerating maximum number of shoot buds from nodal explants of *Stevia* (Jain et al. 2009). Thus, our results showed that BAP in combination with low concentration of IBA was found to be the most effective for shoot bud initiation. In fact, the presence of cytokinins in the medium was essential to induce bud break and shoot proliferation from *Stevia rebaudiana* nodal explants (Thiyagarajan and Venkatachalam 2012).

Multiple shoot bud induction

In order to induce multiple shoots, *in vitro* regenerated shoot buds from nodal explants were cultured on MS medium supplemented with 1 mg l^{-1} BAP in combination with different concentrations (0, 0.25, 0.5 mg l^{-1}) of IBA or IAA. Among the combinations used, IAA (0.25 mg l^{-1}) + BAP (1 mg l^{-1}) and IBA (0.25 mg l^{-1}) +

BAP (1 mg l^{-1}) were the best for multiple shoot bud induction (Figure 1C) with 4.25 and 3.81 shoots/explants, respectively (Table 2).

Table 2: Effect of different concentrations (mg l^{-1}) of IBA and IAA in combination with 1 mg/l BAP on shoot bud multiplication from *in vitro* derived shoot buds of *Stevia rebaudiana* (Bert.).

Growth regulators (mg l^{-1})			No. of	Shoot length
BAP	IBA	IAA	shoots/explant	(cm)
-	-	-	1.68 bc	2.57 ab
1.00	-	0.25	4.25 a	2.23 ab
1.00	-	0.50	1.18 c	0.95 c
1.00	0.25	-	3.81 a	1.09 bc
1.00	0.50	-	2.25 b	2.28 ab
-	0.50	-	1.37 bc	3.42 a

Mean values within the column followed by the same letter in superscript are not significantly different at P<0.5%.

The shoot length (2.23 cm) produced in MS medium containing 0.25 mg l^{-1} IAA and 1 mg l^{-1} BAP was significantly higher than MS medium was supplemented with 0.25 mg l^{-1} IBA and 1 mg l^{-1} BAP (1.09 cm) (Table 2). Our results are in agreement with those reported by Thiyagarajan and Venkatachalam (2012) who found that BAP and IAA combination was superior for induction of shoot bud multiplication from nodal explants of *Stevia* without producing any callus in the culture in comparison to BAP and IBA combination. However, Preethi et al. (2011a) obtained the maximum number of shoots (10.4) from leaf explants of *Stevia* on the MS medium supplemented with BA (1.0 mg l^{-1}), Kn (0.5 mg l^{-1}) and IAA (0.1 mg l^{-1}) whereas the maximum shoot length (4.23 cm) was recorded on the MS medium contained BA (2.0 mg l^{-1}), Kn (0.5 mg l^{-1}) and IAA (0.1 mg l^{-1}).

On the other hand, the number of shoot buds per culture was declined when the IAA or IBA concentration was 0.5 mg l^{-1} in combination with 1 mg l^{-1} BAP in the MS medium. Indeed, the lower number (1.18) as well as the lower length (0.95 cm) of multiple shoots buds per explant was noticed on a medium contained 1 mg l^{-1} BAP and 0.50 mg l^{-1} IAA (Table 2). Our results are in contrast with previous papers which reported that BAP (1.0 mg l^{-1}) and IAA (0.5 mg l^{-1}) combination was found to be the best for multiple shoot bud induction from nodal explants of *Stevia rebaudiana* (Anbazhagan et al. 2010; Thiyagarajan and Venkatachalam 2012). On the contrary, Rafiq et al. (2007) reported that the maximum shoot formation from nodular stem sections of *Stevia rebaudiana* was obtained by supplementing only 2.0 mg l^{-1} BAP in the MS medium.

Besides, when shoot buds were placed on MS medium fortified only with 0.5 mg l^{-1} IBA, few multiple shoots (1.37 shoots /explant) with an average length of 3.42 cm were induced (Table 2). It is noteworthy that the absence of BAP in the MS medium supplemented only with 0.5 mg l^{-1} IBA as well as in the control MS medium, lead to a substantial decline in multiple shoot induction. Indeed, BAP was proved to be the most efficient cytokinin for multiple shoot bud regeneration (Thiyagarajan and Venkatachalam, 2012) whereas auxins were found to be most effective for shoot elongation (Bettaieb et al. 2008).

In vitro rooting of elongated shoots and acclimatization

For root induction, elongated shoots were transferred onto MS medium supplemented with various concentrations of IBA or IAA (0, 0.25, 0.5 mg l^{-1}). Shoots produced roots within two weeks of culture and the data were recorded (Figure 1D).

Table 3: Effect of different concentrations (mg l^{-1}) of IBA and IAA on *in vitro* rooting of elongated shoots of *Stevia rebaudiana* (Bert.).

Growth regulators		Rooting	No. of	Root
IBA (mg l^{-1})	IAA (mg l^{-1})	percent	roots/shoot	length (cm)
-	-	58.46 c	2.86 c	0.94 b
0.25	-	76.92 b	7.14 ab	0.67 c
0.50	-	67.69 b	5.15 bc	0.64 c
-	0.25	96.92 a	4.85 bc	1.26 ab
-	0.50	97.00 a	8.90 a	1.70 a

Mean values within the column followed by the same letter in superscript are not significantly different at P<0.5%.

The first root emerged directly from the basal part of the shoots with no intervening callus. The highest percent (97%) of root induction and the maximum number of roots (8.90 roots/shoot) as well as the root length (1.70 cm) were observed on MS medium fortified with

0.50 mg l^{-1} IAA. Additionally, although the rooting percent was higher (96.92%), the number of roots induced declined by half (4.85 roots /shoot) on MS medium contained 0.25 mg l^{-1} IAA as compared to that recorded on MS medium supplemented with 0.50 mg l^{-1} IAA (Table 3).

Our results are consistent with those of Ahmed et al. (2007) who found that the highest rooting percentage (97.66%) and number of roots per shoot (12.10 roots/shoot) in *Stevia rebaudiana* were noted on MS medium fortified with IAA but with less low concentration (0.1 mg l^{-1}). Concerning the effect of IBA on root initiation, our results showed that the IBA concentration (0.25 and 0.5 mg l^{-1}) in the MS medium has not affected significantly neither the rooting percent nor the number and length of roots (Table 3). On the contrary, it has been reported that the best rooting response through indirect and direct shoot regeneration from *Stevia* leaf explants was observed on MS medium supplemented with 0.5 and 2.0 mg l^{-1} IBA, respectively (Preethi et al. 2011a,b).

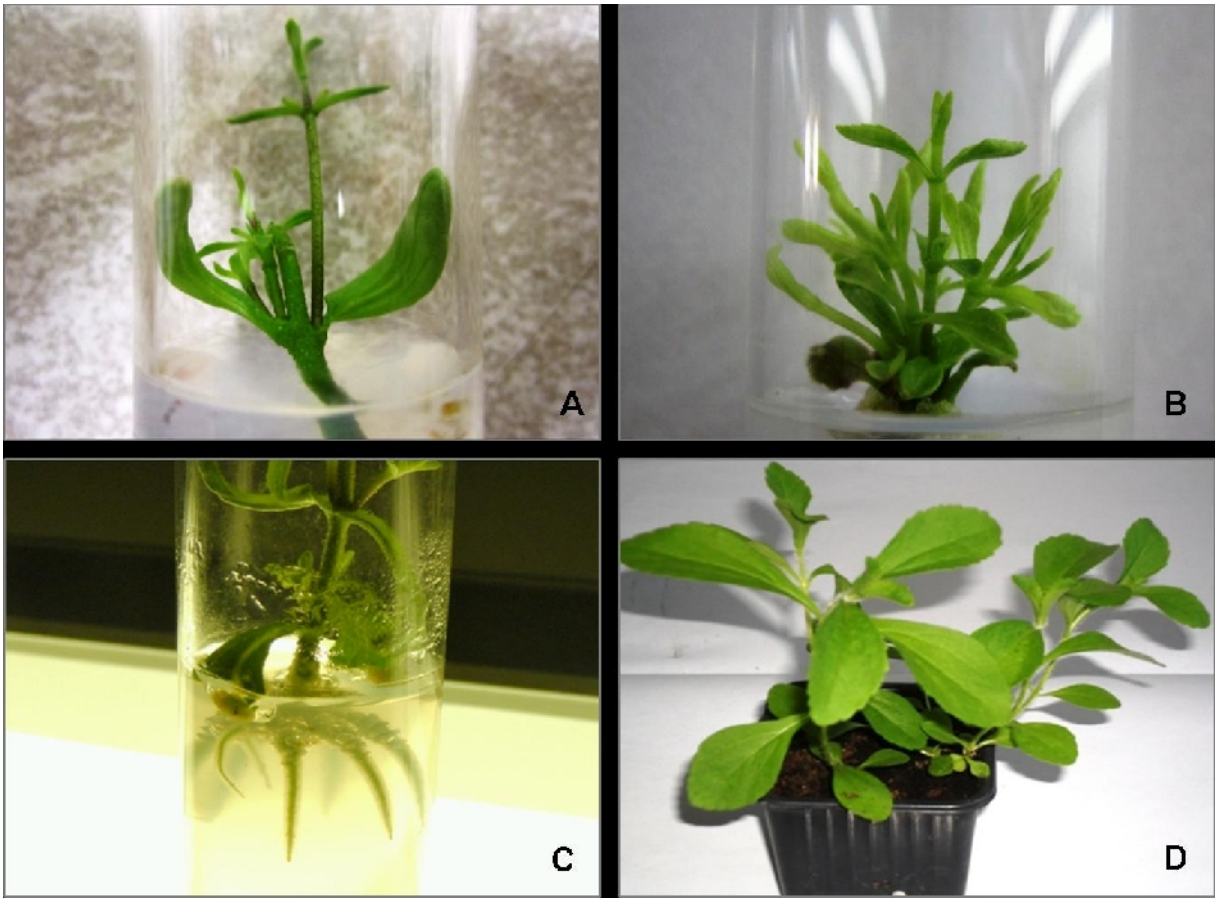

Figure 1: *In vitro* propagation of *Stevia rebaudiana* Bert. (A) Shoot bud initiation; (B) Multiple shoot bud induction; (C) *In vitro* rooting of elongated shoots; and (D) Regenerated plantlet in plastic pot.

In addition, the lowest rooting percent (58.46%) and the minimum number of roots (2.86 roots/shoot) were recorded on the control MS medium (Table 3). In contrast, Ahmed et al. (2007) and Anbazhagan et al. (2010) reported that no rooting of elongated shoots of *Stevia* was observed on auxin-free medium.

Overall, these results showed that auxins enhanced *in vitro* rooting of elongated shoots from nodal explants of *Stevia rebaudiana*. Among the two auxins tested, IAA was found to be superior for rooting in comparison to IBA. The superiority for rooting of IAA over other auxins has also been reported for the same species (Jena et al. 2009; Anbazhagan et al. 2010). Interestingly, roots induced on MS medium supplemented with 0.25 or 0.5 mg l^{-1} IAA were found to be thick and long (≈ 1.5 cm) with fine

roots whereas they were thin and short (≈ 0.6 cm) on MS medium fortified with 0.25 or 0.5 mg l^{-1} IBA (Table 3). In fact, the establishment of an effective root system from *in vitro* shoots is essential for subsequent success during acclimatization of plantlets to autotrophic conditions (Asemota et al. 2007).

The plantlets with well-developed roots were transferred into plastic pots containing peat and were maintained under controlled conditions for two weeks before their transfer into the soil under greenhouse conditions with a survival rate of 67% (Figure 1.D). These plants showed vigorous and uniform growth; they were healthy and similar to donor plants.

Conclusion

The *in vitro* propagation protocol that we have described in this report for direct multiple shoot bud regeneration from nodal explants of *Stevia rebaudiana* is efficient and highly reproducible to get healthy plants in a relatively short period and with a high survival rate. Thus, this protocol could be helpful to establish and cultivate *Stevia* as a promising newly introduced non caloric sweetener and antidiabetic medicinal plant in Tunisia.

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