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IN VITRO PLANTLET PROPAGATION OF *Stevia rebaudiana* BERTONI

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Abstract. *Stevia rebaudiana Bertoni has some kind of diterpenoid steviol glycosides that has no negative effect on blood sugar level. Stevia produces few seeds; therefore, micropropagation is a prevalent method to obtain sufficient amount of uniform and free-disease plants. Most of the previous studies on stevia were emphasized by two steps in vitro shoot or root induction. In the current study, effects of different media containing IAA, IBA and NAA (0.5 and 1 mg·l^{-1}) in combination with BA and kinetin (1 and 2 mg·l^{-1}) on one step cultures of stevia axillary buds were investigated. The results showed that plantlets with a mean shoot length of 8 cm, number of shoots (3.77), number of inter nodes (11.5), fresh weight (0.58 g), dry weight (0.06 g), root length (3.62 cm) and root numbers (12) produce on Murashige & Skoog (1962) medium supplemented with 1 mg.l^{-1} IAA and 1 mg.l^{-1}. The resulted microshoot were superior either for continuing micropropagation or transferring to the pots.*

Keywords*: plant growth regulator, micropropagation, single node culture, Stevia, in vitro culture.*

INTRODUCTION

Stevia rebaudiana Bertoni is an herbal plant belonging to the Asteraceae family. This plant is native to 22°-24° S and 53°-56° W in Paraguay and Brazil (Jain et al. 2009, Sivaram et al. 2003). The native people of Paraguay use it as digestive and superficial ointment (Elkins 1997). Stevia had been used for removing the bitter taste of medicinal plants. The sweetness of stevia is related to the existence of diterpenoid glycosides ent-kaurence. The most important glycosides are stevioside and rebaudioside A (Ramesh et al. 2006, Soejarto 2002). These compounds are 300 times sweeter than sugar and cannot be analyzed or absorbed by human digestive system. Consequently stevia does not have any effects on blood sugar and therefore they are friendly to human health (Taware et al. 2010). This plant could

be used instead of sugar, sugar beet and artificial sweeteners in order to prevent diabetes II (Elkins 1997). Stevia seeds showed low percentage of germination and propagation. Morover, seed does not produce a homogenous population. The resulted plants showed great variety in important characteristics like sweetening level. In addition, due to the low primary growth, the seedling is not able to compete with weeds (Jain et al. 2009). Furthermore, propagation through stem-cutting is limited by stocks of stevia stem, high labor and enough time for obtaining them from single plants (Sivaram et al. 2003). Therfore, propagation through tissue culture could be suitable as an alternative method to obtain sufficient number of plants within short period of time (Ibrahim et al. 2008). There are several reports on proliferation and rooting of stevia using *in vitro* culture method. Hossain et al. (2008) examined the effect of benzyl amino purine (BAP) and kinetin (kin) in different concentrations on shoot multiplication and naphthalene acetic acid (NAA) and indol butyric acid (IBA) on root induction separately. They concluded that 1 $mg.l^{-1}$ BAP was superior on shoot formation and 1.5 $mg.l^{-1}$ NAA produce maximum root formation *in vitro*.

Kumar Jena et al. (2009) reported 2.5 $mg{\cdot}l^{-1}$ IAA was the best case for root production. The results showed that the root formation was faster and roots were longer in 0.5 $mg{\cdot}l^{-1}$ IAA in comparison to IBA. Jain et al. (2009) showed that maximum numbers of shoot buds were obtained in 0.4 $mg{\cdot}l^{-1}$ BAP and 0.5 $mg{\cdot}l^{-1}$ IAA. On the other hand, Taware et al. (2010), reported that the maximum shoot formation is produced in Murashige & Skoog (1962) medium supplemented with 0.3 $mg{\cdot}l^{-1}$ kinetin. While, the maximum root number is obtained in MS medium supplemented with 2 $mg{\cdot}l^{-1}$ IBA.

In the most mentioned reports, the effects of various plant growth regulators (PGRs) were separately examined in 2 steps either for *in vitro* shoot or root formation on stevia. Furthermore, in some previous studies, the growth of stevia was evaluated after 5 or 6 weeks (Taware et al. 2010, Ibrahim et al. 2008). These delays along with producing weak compact shoots are not suitable for plantlet acclimatization. Therefore, the current study investigates various PGRs to introduce a practical method for mass propagation of stevia plantlets in one step and in a shorter period.

MATERIALS AND METHODS

Plant materials and growing conditions

For this experiment stevia pot plants, were kindly provided by Golsaran company, Rasht, Iran. Young and healthy shoots with 10 cm length were obtained from stevia pot plants and transferred to the Guilan university tissue culture laboratory. After

superficial washing of shoots with running water, they were divided into smaller parts and disinfected by applying 1.5% sodium hypochlorite for about 15 minutes. 2-3 drops of Tween 20 was added for reducing adhesion. Thereafter explants were washed in 2, 5 and 10 minutes with distilled sterile water. In order to evaluate appropriate medium for stevia micropropagation, the explants of stevia were cultured on MS medium containing 30% sucrose at pH=5.6 and solidified with 8 $g \cdot l^{-1}$ agar. The medium was supplemented with different concentrations of BA and kinetin each at 1 and 2 $mg \cdot l^{-1}$ in combination with IBA, NAA and IAA at 0.5 and 1 $mg \cdot l^{-1}$, MS medium with free regulator was used for control. The explants were cultured inside the jar glasses containing 40 ml of culture medium with a thin transparent plastic cap that did not have preventing effect on light. The experiment was performed with 10 replications containing 5 explants in each replicate. Glasses were incubated at 25 °C, under 25 μ mol m^{-2} s^{-1} and 16 h photoperiod for 21 days. After 3 weeks, the length of shoot, number of shoot, number of inter node, fresh and dry weight, length and number of roots were measured. Rooted plantlets were transplanted to cup shape pots containing 50% pitmose and 50% perlit and maintend in greenhouse condition incubated at 25 °C, under 16 h photoperiod for 30 days. For decreasing moisture tension, transparent lid was used for 3 days. They were irrigated each 2 dayes for 1 month and then transfered to the field.

Statistical analysis

Collected data were arranged in complete randomized design (CRD) and analysis of variance was accomplished using computer software SAS v. 9.1. and Tukey's Multiple Range Test (DMRT) .

RESULT AND DISCUSSIONS

Shoot growth indicators

Results presented in Table 1 showed that, the maximum shoot length (8.51 cm), shoot number (4.27) and number of inter nodes (12.25) were produced in A4 medium which had no significant difference with A3 medium. For the explants preparation, the distance between two nodes can be suitable for cutting. This emphasizes the importance of inter node numbers instead of the number of nodes because nodes with shorter distance were not suitable for explants preparation. In this study each explant that was grown in A3 or A4 media provided 48 explants with proper inter node distance in 3 weeks. Cultured explants in medium containing BA with IAA, IBA or NAA, produced shoot and root simultaneously. Because of fast growing vigorous shoots with proper roots, the plantlets could be transferred to pots even 2 weeks after culturing (data is not shown). Even though, in this study, the plantlets were transferred after 3 weeks. Therefore, in A3 or A4 medium stevia could be propagated in one step at large level either for mass propa-

Table 1. Effects of different concentrations of PGRs in MS medium on *in vitro* regeneration of *Stevia* from nodal Explants.

Treatment ($mg.l^{-1}$)	Shoot length (cm)	Shoot number (per plants)	Inter node number (per explants)	Root length (cm)	Root number (per explants)	Shoot fresh weight (gr)	Shoot dry weight (gr)
control (MS)	3.37c..g*	1.25 b..f	4.25 e..g	1.5 d...h	3.25 g	0.25 e	0.026 e
A_1 (0.5 IAA+1 BA)	5 c..e	1.61 b..e	7 c..f	2.5 b..d	8 c..f	0.46 b	0.047 c
A_2 (0.5 IAA+2 BA)	5.5 b..d	2.22 b	8 cd	2.72 bc	10 bc	0.48 b	0.049 c
A_3 (1 IAA+1 BA)	8 ab	3.77 a	11.5 ab	3.62 ab	12 ab	0.58 a	0.06 ab
A_4 (1 IAA+2 BA)	8.51 a	4.27 a	12.25 a	4.04 a	13.25 a	0.62 a	0.064 a
B_1 (0.5 IAA+1 kin)	2.8 d..g	0.71 d..f	1.5 h..g	1.2 e..i	3 g	0.12 f	0.012 f
B_2 (0.5 IAA+2 kin)	2.5 e..g	0.4 f	0.5 j	0.5 g..i	1.5 gh	0.10 f	0.01 gf
B_3 (1 IAA+1 kin)	3 c..g	1.02 c..f	2 c..f	1.5 d..h	2.5 g	0.085 gf	0.008 gf
B_4 (1 IAA+2 kin)	3.48 c..g	0.77 d..f	2.5 g..j	0.75 g..i	2 gh	0.12 f	0.01 gf
C_1 (0.5 IBA+1 BA)	4.5 c..e	1.27 b..f	5 d..g	2 c..f	6 f	0.25 e	0.026 e
C_2 (0.5 IBA+2 BA)	4.7 c..e	1.27 b..d	6 c..f	2.25 c..e	7 ef	0.35 c	0.036 d
C_3 (1 IBA+1 BA)	5.28 bcd	2 bc	7.5 cde	2.5 bcd	10 bc	0.48 b	0.05 c
C_4 (1 IBA+2 BA)	5.6 bc	1.83 bcd	8 cd	2.5 bcd	10.25 bc	0.5 b	0.052 bc
D_1 (0.5 IBA+1 kin)	2.6 efg	0.83 c..f	0.62 ij	1 g..i	1 gh	0.1 gh	0.01 gh

Table 1 (continued)

Treatment ($mg.l^{-1}$)	Shoot length (cm)	Shoot number (per plants)	Inter node number (per explants)	Root length (cm)	Root number (per explants)	Shoot fresh weight (gr)	Shoot dry weight (gr)
D_2 (0.5 IBA+2 kin)	3.8 c..f	0.77 d..f	0.12 j	0 i	0 i	0.055 g..j	0.005 gh
D_3 (1 IBA+1 kin)	3 c..g	1.02 c..f	1 hij	0.75 ghi	1.5 gh	0.12 f	0.012 f
D_4 (1 IBA+2 kin)	2.85 c..g	0.77 d..f	0.5 j	0.5 hi	1 gh	0.085 gfh	0.008 gh
E_1 (0.5 NAA+1 BA)	4 c..f	1.27 b..f	4 g..f	1.5 d..f	6.25 ef	0.33 cd	0.035 de
E_2 (0.5 NAA+2 BA)	4.4 cde	1.25 b..f	6.5 c..f	1.7 def	7.5 ef	0.28 de	0.029 de
E_3 (1 NAA+1 BA)	5.2 cde	1.32 b..f	7 c..f	2.37 cde	8.5 cde	0.35 c	0.036 d
E_4 (1 NAA+2 BA)	5.5 bcd	1.67 bcd	8.5 bc	2.25 cde	9.5 cd	0.45 b	0.046 c
F_1 (0.5 NAA+1 kin)	2.5 efg	0.9 c..f	1.15 hij	0.5 hi	1.25 gh	0.02 ij	0.0025 g
F_2 (0.5 NAA+2 kin)	1.01 g	0.52 ef	0.25 j	0 j	0 h	0.07 j	0.0007 g
F_3 (1 NAA+1 kin)	2.7 d..g	1.4 b..f	1.12 hij	0.53 ghi	1.5 gh	0.065 ghi	0.0067 fg
F_4 (1 NAA+2 kin)	1.56 fg	0.75 def	0.5 j	2 i	0.95 gh	0.045 hij	0.0046 fg

* For each parameter significant difference between mean
among the sites are indicated by different letters (Tukey test, alpha = 0.05).

gation or for transferring to field. Debnath (2008) reported that the maximum shoot proliferation was in 2 mg.l-1 BAP with 1.13 mg.l-1 IAA. Minimum shoot length (1.01) with minimum shoot number (0.52) and minimum number of inter node (0.25) was obtained in F2 medium. Maximum fresh (0.62 g) and dry weight (0.064 g) were resulted in A4 (Table 1), which had no significant differences with A3. High amount of dry weight may be due to high level of carbohydrate accumulation in plant structure. As a result, plants with high amount of carbohydrate had more resistance in the acclimatization period (Ramesh et al. 2006).

Root formation Index

Maximum root length (4.04 cm) and root number (13.25) were produced in MS medium supplemented with 1 $mg.l^{-1}$ IAA and 2 $mg.l^{-1}$ BA (A4) that shows no significant difference with A3 in root length (3.62 cm) and root number (12), respectively (Table 1). The minimum root length was observed in F2 treatment. Both F2 and F4 media were contained 2 $mg.l^{-1}$ kinetin, but growth of shoot and root in F4 was more than F2 and was similar to B2 and B4 (Table 1). The increase in auxin concentration from 0.5 to 1 $mg.l^{-1}$ modified improper effects of kinetin. Similar results have been reported by Jena et al. (2009).

Orthogonal comparison of treatments

According to the results obtained among different groups of A, B, C, D, E and F in Table 1, orthogonal comparison between group A and other groups (Table 2) showed that media containing different concentration of IAA and BA could produce longer shoots, more shoot numbers, more fresh and dry weights, longer roots and more root numbers compare to others. As a result, IAA and BA combination was more suitable for stevia propagation than other used combinations. Sivaram et al. (2003) reported that combination of IAA and BA was proper for shoot formation of stevia in *in vitro* culture, too. Also, orthogonal comparison between groups A, C and E with groups B, D and F showed (Table 3) that stevia explants grown in medium containing kinetin (B, D and F) produced shorter shoots, roots and inter node numbers than explants grown in medium containing BA (A, C and E).

The presence of kinetin in medium especially 2 $mg.l^{-1}$ caused callus formation with limited shoot and root formation (Fig. 1). As a result, regarding the mean and orthogonal comparison (Table 3), the treatments containing BA hormone were more suitable than those containing kinetin. The reason is that, kinetin in stevia medium tends to cause more calli and subsequently

Table 2. Orthogonal comparison among groups of media for *in vitro* regeneration of *Stevia* from nodal explant.

Treatment ($mg.l^{-1}$)	Shoot length (cm)	Shoot number (per plants)	Inter node number (per explants)	Root length (cm)	Root number (per explants)	Shoot fresh weight (gr)	Shoot dry weight (gr)
IAA+BA	6.62 a*	2.96 a	9.68 a	3.22 a	10.81 a	0.53 a	0.055 a
IAA+ kin IBA+BA IBA+ kin NAA+BA NAA+ kin	3.54 b	1.07 b	3.41 b	1.46 b	4.44 b	0.19 b	0.027 b

* For each parameter significant difference between mean among the sites are indicated by different letters (Tukey test, alpha = 0.05).

Table 3. Orthogonal comparison among treatments containing BA and kinetin for *in vitro* regeneration of stevia from nodal explant.

Treatment ($mg.l^{-1}$)	Shoot length (cm)	Shoot number (per plants)	Inter node number (per explants)	Root length (cm)	Root number (per explants)	Shoot fresh weight (gr)	Shoot dry weight (gr)
IAA+BA IBA+BA NAA+BA	5.51 a*	2.04 a	7.6 a	2.5 a	9.02 a	0.42 a	0.044 a
IAA+ kin IBA+ kin NAA+ kin	2.65 b	0.82 b	0.98 b	0.97 b	1.62 b	0.082 b	0.022 b

* For each parameter significant difference between mean among the sites are indicated by different letters (Tukey test, alpha = 0.05).

shoot and root formations were prevented. This leads to the production of mal-form plantlet with unsuitable characteristics for transplanting. With agreement to our results, Hossain et al. (2008) and Shatnawi et al. (2011) reported that BAP had better effect than kinetin for increasing the number of shoots and length of stevia. The A group of treatments containing IAA+BA was more effective than others on shoot and root formation. In *in vitro* propagation of stevia, the combination of IAA+BA was better than IAA+ Kin, IBA+BA, IBA+ Kin, NAA+BA and NAA+ Kin (Table 2). All the *in vitro* characters measured were significantly larger in BA than kinetin (Table 3).

Figure 1. Stevia plantlets grown on the presence of kinetin.

Maximum shoot and root formation were observed on A4 medium (1 $mg.l^{-1}$ IAA+2 $mg.l^{-1}$ BA) that had no significant difference with A3 (1 $mg.l^{-1}$ IAA+1 $mg.l^{-1}$BA). Consequently A3 (1 $mg.l^{-1}$ IAA+1 $mg.l^{-1}$BA) appeared to be the best component for 1 step micropropagation of complete plantlets of stevia. These plantlets were most suitable either for further multiplication or for transplanting to pot (Fig. 2). Furthermore, *in vitro* derived plants had shown high potential for acclimatization; so more than 97% of plantlets survived when they were transferred to *ex vitro* conditions. Shatnawi et al. (2011) achieved A survival of 90% when rooted explants were acclimatized *in vivo* in 1 soil: 1 perlite: 1 peat.

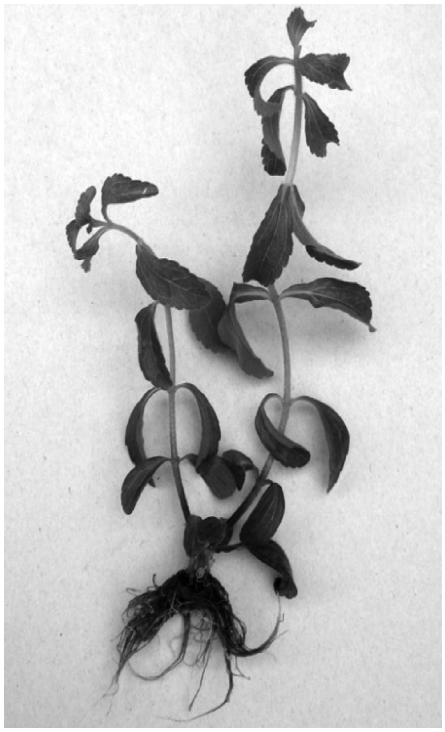

Figure 2. Stevia plantlet grown on 1 mg.l^{-1} IAA+2 mg.l^{-1}BA medium (A3)

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