Pradhan and Dwivedi, Vegetos 2016, 29:4
10.5958/2229-4473.2016.00102.6

Vegetos- An International Journal of Plant Research

Research Article A SCITECHNOL JOURNAL

In vitro Shoot multiplication of *Stevia rebaudiana*, an important Plant with high Economic and Medicinal values

Navin Pradhan and Padmanabh Dwivedi*

Abstract

Stevia rebaudiana (Bertoni), known to be an anti-diabetic medicinal herb, belongs to Asteraceae family which has bio-active compounds stevioside and rebaudioside in its leaves that taste about 300-350 times sweeter than sucrose. Nodal segments were cultured in half-strength Murashige and Skoog (MS) medium supplemented with kinetin (Kn) and benzylaminopurine (BAP), alone and in combination (0.2, 0.5, 1.0, 2.0 and 3.0 mg L^{-1}), for shoot proliferation while various concentrations of auxins i.e. indole acetic acid (IAA) and indole butyric acid (IBA) (0.2, 0.5, 1.0, 2.0 and 3.0 mg L^{-1}), alone or in combination with different cytokinins, for rooting. Almost in all the cultures shoot primordia initiation was observed 2-5 days after the inoculation. Overall, from the economical point of view, half-strength MS media with 0.2 mg L^{-1} Kn was the best producing maximum number of shoot (3.00 ± 0.00), shoot length (7.13 ± 0.43) and number of leaves (27.75±0.85), after 60 days of inoculation. However, after 30 days of transfer in rooting media supplemented with combined IBA (2.0 mg L^{-1}) and Kn (0.5 mg L^{-1}) showed best response in number of roots (10.00 ± 0.41) and length of root (cm) (6.73 ± 0.09), respectively. Regenerated plantlets were hardened in potting mixture containing perlite: sand: soil (1:1:1) for 2 months inside culture room with 70% relative humidity and later transferred in green house for 8 weeks before final transfer into the field. This micro-propagation method using half-strength MS media could be economical and effectively used for mass production of *Stevia rebaudiana* under *in vitro* condition.

Keywords

Diabetes; Micropropagation; Murashiage and skoog medium; Nodal explants; Stevia

Introduction

Stevia rebaudiana (Bertoni) is known to be an anti-diabetic medicinal herb belonging to Asteraceae family, which has gained more importance in recent years for its economic and medicinal virtues. Besides this, it has other remarkable medicinal properties such as anti-hyperglycaemic, anti-cancerous [1,2], treatment of diabetes mellitus, obesity, caries prevention [3], regulates blood pressure [4] and anti-hypersensitive [5,6]. It is well adapted to local conditions and normally requires mild temperature and relative humidity (80%) [4,7]. It is indigenous to South America-Brazil and Paraguay [8]. It is a natural sweetener herb, estimated about 300 times sweeter than sucrose [9], due to its active constituents of diterpene glycosides i.e. stevioside and rebaudioside present in its leaves. The seeds of stevia show very low (<10%) germination rates [10,11]. Moreover, production of homogeneous populations could not be achieved due to self-incompatibility of seeds [12], resulting in variability in sweetener level and composition. Propagation by vegetative means through stem cuttings is also limited by low number of individuals that could be obtained from single plant. These constraints limit the growth and development leading to poor cultivation and difficulties for its multiplication and establishment in the field. Therefore, this requires the basic micro-propagation method for proliferation of plant materials with high multiplication rates. Tissue culture offers rapid micropropagation method for regeneration of genetically true-to-type plants on large scale within a short period of time.

Previous studies reported that various medicinal plants have been propagated through micropropagation [13]. In addition, several reports suggested that propagation of *Stevia rebaudiana* through tissue culture [14,15,16] has significance commercial value. Thus, large scale production of healthy plantlets was made possible by meristem or shoot tip culture [1,17], nodal segments [16,18], inter-nodal segment [19,20], callus [16], leaf [21], which seems to be successful for commercial micropropagation of stevia. However, maximum research for *in vitro* propagation of stevia using different explants in full-strength MS medium supplemented with various plant growth regulators have been reported [22]. But limited research was found in half-strength MS medium under *in vitro condition* [23,24]. Yet there is ample scope for propagation of stevia using different plant growth regulators in half-strength MS medium since it is more economical. Therefore, the main objective of the present study was to find out the best combination of plant growth regulators for multiplication and establishment of a reliable protocol for *in vitro* regeneration of *Stevia rebaudiana* using half-strength MS media.

Materials and Methods

Culture initiation

The young healthy profusely growing plants free from disease symptoms were collected from local Herbal nurseries and established in Horticulture garden of Institute of Agricultural Sciences, BHU, India, and used as a source of explants. Shoots were not more than 2-3 months old, nodal segments were used as explants with 1.5-2.0 cm long and 0.2-0.5 cm thick. Explants were thoroughly washed in running tap water for 10 min. Afterwards the explants were again washed with 2-3 drops of Tween 80 (Himedia Laboratories, India) for 8-10 min followed by 4-5 times washing with double distilled water. Explants were then taken into laminar air flow cabinet for further surface sterilization with Bavistin 0.002% (w/v) for 4-5 min. Thereafter explants were treated with 0.1% (w/v) mercuric chloride ($HgCl_2$) for 1 min. Afterwards the explants were thoroughly washed 4-5 times with distilled water to get rid of any traces of chemicals.

Shoot multiplication

Nodal explants were cultured on half-strength MS media supplemented with kinetin (Kn) and benzylaminopurine (BAP),

***Corresponding author:** Padmanabh Dwivedi, Laboratory of Plant Tissue Culture and Stress Physiology, Department of Plant Physiology, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi, India, Tel: 0542-6701311; E-mail: pdwivedi25@rediffmail.com

Received: September 09, 2016 **Accepted:** October 12, 2016 **Published:** October 14, 2016

alone and in combination (0.2, 0.5, 1.0, 2.0 and 3.0 mg L^{-1}), for shoot multiplication, and parameters such as days taken for shoot primordia initiation (DPI), number of shoot per explant, length of shoot (cm), number of leaves were recorded at 20, 40 and 60 days after inoculation (DAI) except DPI, as it was observed up to 2-8 DAI. All the media contained 15 g L^{-1} sucrose, 8 g L^{-1} agar while pH was adjusted to 5.8 before autoclaving at 15 psi (121°C) for 25 minutes.

Rooting and acclimatization

For root initiation, number of root per explant and length of root (cm) were recorded after 30 days of culture in rooting media which was supplemented with indole acetic acid (IAA) and indole butyric acid (IBA) with varying concentrations i.e. 0.2, 0.5, 1.0, 2.0 and 3.0 mg L^{-1}), applied singly or in combination of cytokinins (Kn, BAP and BAP+Kinetin), in half-strength MS medium. Well rooted *in vitro* regenerated plants were washed gently by sterilized water to remove agar adhered on the roots and transferred to plastic cups containing sterile mixture of soil and perlite (2:1) for acclimatization. The plantlets were covered by transparent polythene and kept under 70% relative humidity at 25 ± 2°C with 16: 8 h (light: dark) photoperiod. After 6 weeks of acclimatization, the plantlets were then transferred to green house for hardening under normal day length condition.

Culture conditions

All the cultures were incubated under 70-80% relative humidity with 16 : 8 h (light : dark) photoperiod in an air-conditioned culture room at 25 ± 2°C provided by cool fluorescent tube lights with 50 µMol m^{-2} s^{-1} light intensity.

Data analysis

Data were analyzed by one-way analysis of variance (ANOVA) using the SPSS software (version 16.0). There were four replicates in each treatment, and each experiment was repeated thrice. All the data were presented in the form of mean ± standard error mean (SEM). Means were compared for significant difference assessed by Duncan's Multiple Range Test (DMRT) at $P \leq 0.05$ [25].

Results and Discussion

Days taken for primordia initiation (DPI)

The response of different concentrations of kinetin, BAP and BAP+kinetin was observed on number of days taken for primordia initiation after inoculation (DPI) (Figure 1). The treatment T1 (0.2 mg L^{-1}) with kinetin (1.50 ± 0.29) significantly took less number of days for primordia initiation when compared to control (T0) (3.75 ± 0.48). While both treatments T1 (0.2 mg L^{-1}) and T2 (0.5 mg L^{-1}) took same number of days for initiation of shoot primordia (2.00 ± 0.00) with BAP treatment. The combined effect of BAP+kinetin for DPI showed that treatment T0 and T2 (0.5 mg L^{-1}) had taken lesser time for the initiation of shoot primordia as compared to T1, T4, T3, T5. Among all the PGRs tried, kinetin with 0.2 mg L^{-1} performed better as compared to rest of the treatments for the initiation of shoot primordia in *Stevia rebaudiana* under *in vitro* condition.

Early bud break was observed in medium supplemented with single use of kinetin, T1 (0.2 mg L^{-1}) i.e., about 2 days taken for primordia initiation after inoculation as compared to control (Figure 1). The stimulating effect of kinetin and BAP or in combination on early bud break and multiple shoot formation has been reported earlier in several medicinal and aromatic plant species including *Chlorophytum borivilianum* [26], *Oscimum spp.* [27], *Withania somnifera* [28] and *Stevia rebaudiana* [16,23,24,29,30].

Number of Shoots Per Explant

The data on number of shoots (NOS) per explant recorded at 20, 40 and 60 DAI in *Stevia rebaudiana* under *in vitro* condition in half strength MS media are presented in Figure 2. The number of shoot per explant was observed with different concentrations of kinetin, BAP along with their combinations at different DAI. It was found that kinetin showed maximum number of shoot per explant with treatment T1 (0.2 mg L^{-1} kinetin) i.e., 2.50 ± 0.29, 2.50 ± 0.29 and 3.00 ± 0.00 at 20, 40 and 60 DAI, respectively, while it was minimum with treatment T5 (3 mg L^{-1} kinetin) with similar values of 1.7 5 ± 0.25 at all the three stages i.e. 20, 40 and 60 DAI. When the NOS per explant was observed with BAP, it was found that among all the stages, treatments T0, T1 and T2 responded similar to kinetin with the highest value (i.e. 2.00 ± 0.00) as compared to treatment T3, T4 and T5 (1.75 ± 0.25, 1.50 ± 0.29 and 1.50 ± 0.29) at 20, 40 and 60 DAI, respectively. The effect of both BAP+Kinetin was also observed, and it was found that treatment T0 (considered as control), T1 (0.2 mg L^{-1} BAP+Kinetin) and T2 (0.5 mg L^{-1}) produced higher number of shoot per explant (i.e. 2.00 ± 0.00) in all the three concentrations as compared to rest

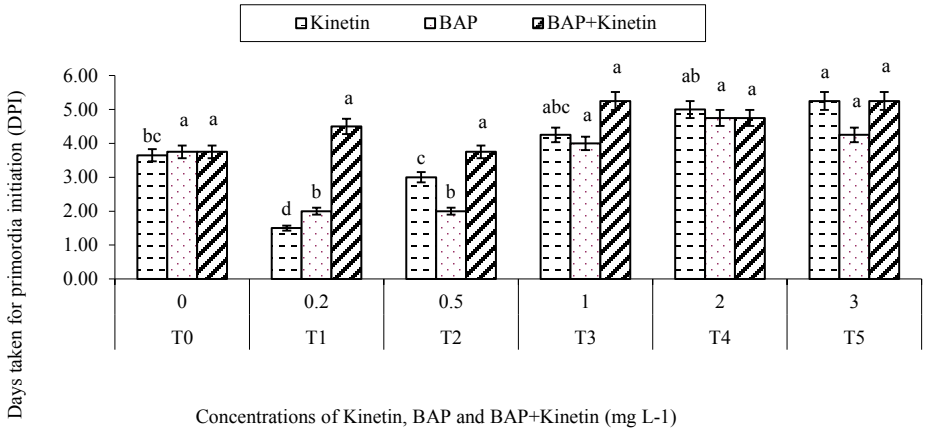

Figure 1: Effect of Kinetin and BAP along with their combinations on number of days taken for initiation of shoot primordia after inoculation in *Stevia rebaudiana* under *in vitro* condition in half strength MS media. Data have been recorded after a week of inoculation. Data are in the form of mean±SEM, and means followed by the same letters within the columns are not significantly different at P≤0.05 using Duncan's multiple range test. BAP=6-Banzyl amino purine, DAI=Days after inoculation, DPI= Days taken for primordia initiation.

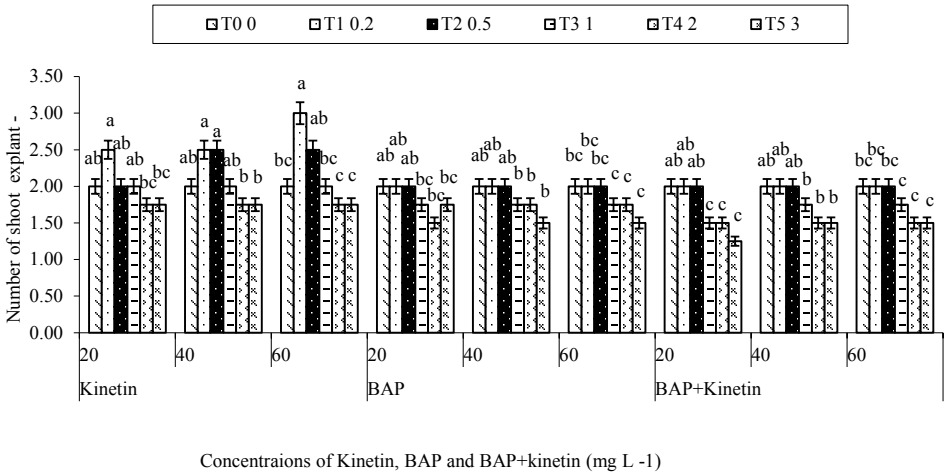

Figure 2: Comparative effect of different concentrations of Kinetin and BAP along with their combinations on number of shoots explant^{-1} in *Stevia rebaudiana* under *in vitro* condition in half strength MS media, at 20, 40 and 60 DAI. Data are in the form of mean ± SEM, and means followed by the same letters within the columns are not significantly different at P ≤ 0.05 using Duncan's multiple range test. BAP=6-Banzyl amino purine, DAI=Days after inoculation.

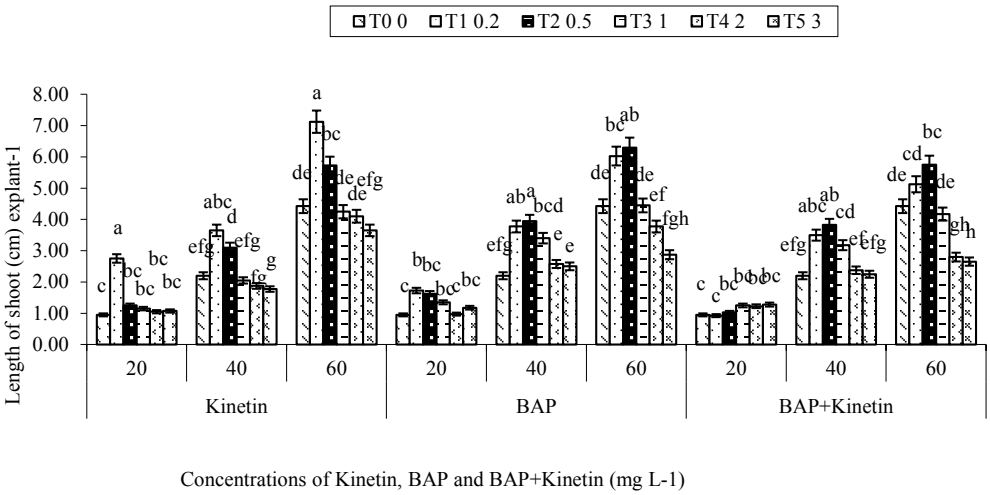

Figure 3: Comparative effect of different concentrations of Kinetin and BAP along with their combinations on length of shoot (cm) explant^{-1} in *Stevia rebaudiana* under *in vitro* condition at 20, 40 and 60 DAI. Data are in the form of mean±SEM, and means followed by the same letters within the columns are not significantly different at P≤0.05 using Duncan's multiple range test. BAP=6-Banzyl amino purine, DAI=Days after inoculation.

of the treatments. Among all the PGRs tried, kinetin with treatment T1 (0.2 mg L^{-1}) performed better in terms of NOS per explant at all the three stages of inoculation as compared to BAP and BAP+kinetin. Data were significant at 5% level of significance.

Growth regulators have the potential to alter almost all aspects of plant growth and development both as promoter as well as retardant, when used in appropriate concentration. It is a general observation that lower concentration of kinetin (considered only in half strength) is better in performance than BAP and BAP+kinetin for multiple shooting, length of shoot. In our study it is to be noted that among different growth regulators applied alone or in combination with varying concentrations of kinetin, BAP and BAP+kinetin, maximum number of shoot per explant was found at 60 DAI in half strength MS media supplemented with kinetin alone i.e. T1 (0.2 mg L^{-1}), about 50% more when compared to control (basal) treatment (Figure 2). These results are in agreement with other studies [16,31-35]. The increased shoot number may be due to suppression of apical dominance during subculture that induced basal dormant meristematic cells to form new shoots [36].

Length of Shoot (cm) Per Explant

The length of shoot explant^{-1} was observed with different levels of kinetin, BAP and BAP+Kinetin at different DAI (Figure 3). Maximum length of shoot explant^{-1} (7.13 ± 0.43 cm) was observed with kinetin T1 (0.2 mg L^{-1}), while it was minimum (3.65 ± 0.32 cm) with treatment T5 at 60 DAI as compared to control (T0). However, among all the treatments, control without stress i.e. (T0) showed lesser length of shoot explant^{-1} (0.95 ± 0.33 cm) at 20 DAI. The effect of BAP was observed on length of shoot per explant: maximum length was observed with treatment T1 (1.73 ± 0.21 cm) at 20 DAI and T2 (3.95 ± 0.31 and 6.30 ± 0.30 cm) at 60 DAI, whereas, it was minimum with T0 (i.e. 0.95 ± 0.33, 2.20 ± 0.11 and 4.43 ± 0.20 cm) and T5 (i.e. 1.18 ± 0.26, 2.50 ± 0.19 and 2.88 ± 0.18 cm) at 20, 40 and 60 DAI, respectively. The combined effect of BAP + kinetin was also observed

at different DAI, with treatment T2 producing higher length of shoot (5.75 ± 0.27 cm) at 60 DAI. Among all the PGRs, kinetin performed better on increasing length of shoot with treatment T1 (0.2 mg L^{-1}) at 60 DAI.

In the present investigation, data revealed that increment in number of shoot per explant was found maximum at 60 DAI in half strength MS media supplemented with kinetin alone i.e. T1 (0.2 mg L^{-1}), about 60.8% more as compared to control (basal) (Figure 3). This observation is similar to those reported by Mehta et al. [33]; Singh and Dwivedi [35]; Singh et al. [24].

Number of Leaves Per Explant

The number of leaves per explant was observed with different levels of kinetin, BAP and BAP+kinetin at 20 days interval i.e. at 20, 40 and 60 DAI as illustrated in Figure 4. Maximum number of leaves per explant i.e., 14.50 ± 1.19, 19.75 ± 1.75 and 27.25 ± 0.88 was observed with kinetin 0.2 mg L^{-1} at 20, 40 and 60 DAI, respectively. However, minimum number of leaves per explant i.e., 6.00 ± 0.41, 10.50±0.65 and 14.50 ± 0.65 was found with treatment T5 (3 mg L^{-1}). With BAP, it was found that treatment T1 (0.2 mg L^{-1}) increased the number of leaves per explant along with the increasing DAI and was maximum 22.75±0.63 at 60 DAI. The data significantly increased in increasing order along with different days of interval at 20, 40 and 60 DAI. The minimum number of leaves was found with treatment T0 (6.50 ± 1.50) at 20 DAI and with treatment T5 (12.00 ± 0.71 and 13.50 ± 0.87) at 40 and 60 DAI, respectively. Similarly, the combined effect of BAP+kinetin produced maximum number of leaves (20.25 ± 0.48) with treatment T1 (i.e. 0.2 mg L^{-1} BAP+kinetiin) while minimum (11.00±0.41) with treatment T4 (2 mg L^{-1}). However, among all the PGRs, kinetin (0.2 mg L^{-1}) performed better in increasing number of leaves as compared to BAP and BAP+kinetin. Data were significant with different treatments along with different stages of inoculation.

It was noted that highest percentage of response was shown with respect to number of leaves per explant at 60 days after inoculation in half strength MS media supplemented with kinetin alone i.e. T1 (0.2 mg L^{-1}), with about 54.2% more response as compared to control (Figure 4). Increase in number of leaves might be due to increase in meristematic activity of explant leading to more number of shoots. These results are supported by Mehta et al. [33]; Ranganathan [34]; Singh et al. [16]; Singh and Dwivedi [24]; Singh et al. [35].

Effect of IBA, Kinetin, BAP and their Combination on In vitro Root Induction

It is well a known fact that IBA influences root formation. The number of root (NOR) and length of root (RL) expressed in cm were significantly influenced by IBA and its combination with kinetin, BAP and BAP+kinetin in *Stevia rebuadiana* under *in vitro* condition. The *in vitro* raised plantlets were re-inoculated in one fourth strength MS media supplemented with different concentrations of IBA, kinetin and BAP+kinetin for root induction (Table 1, Figure 5). Both NOR and RL were higher with IBA treatment T3 i.e. 8.25 ± 0.25 and 6.38 ± 0.07 cm), respectively, while the values of these parameters were lesser with treatment T1 (3 mg L^{-1}) and T0 (control) i.e. , 4.25 ± 0.48 and 2.08 ± 0.15 cm), respectively, under *in vitro* condition, after 30 days of culture in rooting media. The IBA with treatment T1 (3 mg L^{-1}) significantly reduced the NOR and RL when compared with rest of the IBA concentrations.

Further, different levels of IBA were applied in combination of kinetin, BAP and BAP+kinetin; it was observed that treatment T2 showed significantly higher NOR i.e., 10.00 ± 0.41, 6.50 ± 0.09 and 6.75 ± 0.48), respectively. Similar pattern was also observed with root length (cm) i.e., (6.73 ± 0.48, 6.33 ± 0.09 and 5.85 ± 0.26), respectively. Though, minimum NOR i.e., (4.25 ± 0.25, 3.50 ± 0.29 and 3.75 ± 0.48) was observed with treatment T5 for IBA, IBA+kinetin, IBA+BAP and IBA+BAP+kinetin, respectively under *in vitro* conditions. Whereas, minimum RL (2.08 ± 0.15) was found in both treatments with T0 (control) of IBA+kinetin and IBA+BAP, respectively.

From the present study, it is clear that highest percentage of response for root induction using different combinations of IBA with Kinetin, BAP and BAP+kinetin under *in vitro* condition was found to be about 2.2 and 3.5 folds more with treatment T2 (IBA+kinetin @ 2+0.5 mg L^{-1}) for number of roots and length of roots, respectively, as compared to control (basal) (Table 1, Figure 5). Similar findings have been reported by Singh et al. [16]; Singh and Dwivedi [23]; Singh and Dwivedi [24]; Deng et al. [37]; Bayoudh et al. [38].

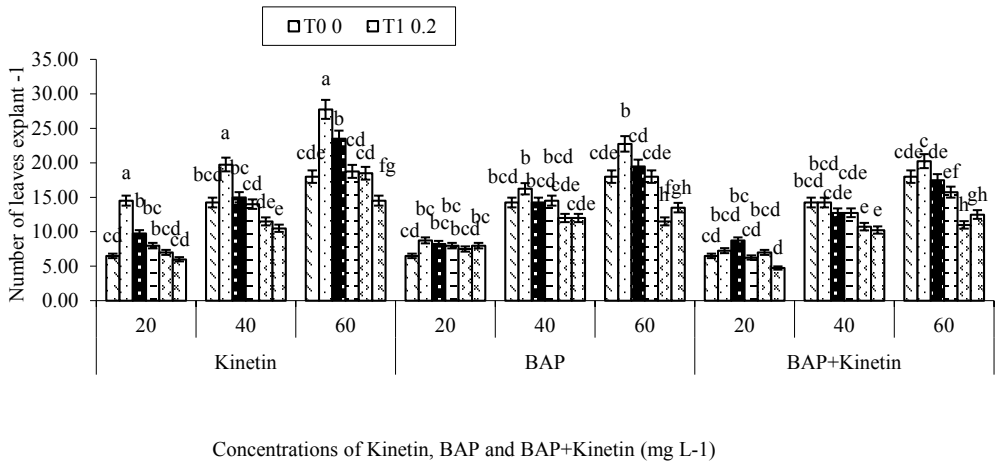

Figure 4: Comparative effect of different concentrations of Kinetin and BAP along with their combinations on number of leaves explant $^{-1}$ in *Stevia rebaudiana* under *in vitro* condition. Data are in the form of mean ± SEM, and means followed by the same letters within the columns are not significantly different at P≤0.05using Duncan's multiple range test.

Table 1: Comparative effect of IBA, Kinetin, BAP and their combinations on *in vitro* root induction in *Stevia rebaudiana*.

Treatments	Plant growth regulator (mgl^{-1})				Number of roots	Length of root (cm)
T0	IBA	0			4.50 ± 0.29^{c}	2.08 ± 0.15^{e}
T1		3			4.25 ± 0.48^{c}	3.33 ± 0.23^{d}
T2		2			6.50 ± 0.29^{b}	5.88 ± 0.27^{ab}
T3		1			8.25 ± 0.25^{a}	6.38 ± 0.07^{a}
T4		0.5			6.50 ± 0.29^{b}	5.55 ± 0.24^{b}
T5		0.2			6.00 ± 0.41^{b}	4.40 ± 0.24^{c}
T0	IBA	0	Kinetin	0	4.50 ± 0.29^{d}	2.08 ± 0.15^{d}
T1		3		0.2	5.25 ± 0.25^{c}	3.50 ± 0.19^{c}
T2		2		0.5	10.00 ± 0.41^{a}	6.73 ± 0.09^{a}
T3		1		1	7.25 ± 0.48^{b}	5.68 ± 0.36^{b}
T4		0.5		2	4.50 ± 0.29^{c}	3.48 ± 0.25^{c}
T5		0.2		3	4.25 ± 0.25^{c}	2.65 ± 0.12^{d}
T0	IBA	0	BAP	0	4.50 ± 0.29^{d}	2.08 ± 0.15^{c}
T1		3		0.2	5.00 ± 0.41^{b}	3.23 ± 0.16^{b}
T2		2		0.5	6.50 ± 0.29^{a}	6.33 ± 0.09^{a}
T3		1		1	6.00 ± 0.41^{a}	6.03 ± 0.13^{a}
T4		0.5		2	4.50 ± 0.29^{b}	3.65 ± 0.23^{b}
T5		0.2		3	3.50 ± 0.29^{c}	2.25 ± 0.06^{c}
T0	IBA	0	BAP+Kinetin	0	4.50 ± 0.29^{c}	2.08 ± 0.15^{c}
T1		3		0.2	4.50 ± 0.29^{b}	2.98 ± 0.17^{b}
T2		2		0.5	6.75 ± 0.48^{a}	5.85 ± 0.26^{a}
T3		1		1	6.50 ± 0.29^{a}	5.60 ± 0.21^{a}
T4		0.5		2	4.50 ± 0.29^{b}	2.85 ± 0.13^{b}
T5		0.2		3	3.75 ± 0.48^{b}	2.03 ± 0.09^{c}

where, IBA=Indole-3-butyric acid, BAP=6-Benzyl amino purine. Parameters have been recorded after 4 weeks (30 days) of transfer in rooting media. Data are in the form of mean ± SEM, and means followed by the same letters within the columns are not significantly different at P ≤ 0.05 using Duncan's multiple range test.

Effect of IAA, Kinetin, BAP and their Combination on In vitro Root Induction

The number of roots and length of root (cm) both are significantly influenced by IAA and its combination with kinetin, BAP and BAP+kinetin in *Stevia rebaudiana* under *in vitro* condition in one fourth strength of MS media (Table 2). Both NOR and RL were higher in treatment T3 (1 mg L^{-1} IAA) i.e., 6.50 ± 0.29 and 6.00 ± 0.07, respectively, while the lesser values were noticed with treatment T1 (3 mg L^{-1} IAA) having 3.50 ± 0.29 NOR, and T0 (0 mg L^{-1} IAA) with 2.08 ± 0.15 RL. Accordingly, the highest number of roots (7.50 ± 0.29) and length of roots (cm) (5.58 ± 0.11) were observed in T2 when IAA (2 mg L^{-1}) was combined with kinetin (0.5 mg L^{-1}), as compared to all the rest of the IAA concentrations. The lowest number of roots was observed in treatment T5 (0.2 mg L^{-1}) i.e., 3.50 ± 0.29 while minimum length of root was observed in treatment T0 (2.08 ± 0.15).

When IAA was combined with BAP (1 mg L^{-1}), highest number of roots (6.50 ± 0.29) was observed in treatment T3 while maximum length of root (cm) was produced with treatment T4 (5.23 ± 0.19). The minimum NOR and RL was observed with treatment T5 and T0, respectively. In combined treatment of IAA with BAP+kinetin, treatment T2 showed maximum NOR as well as RL (6.00 ± 0.58 and 5.38 ± 0.13), whereas minimum NOR was observed similar with treatment T1, T4 and T5 (3.50 ± 0.29), furthermore, minimum RL (2.08 ± 0.1) was observed with treatment T0 and T5.

where, IAA=Indole-3-acetic acid, BAP=6-Benzyl amino purine. Parameters have been recorded after 4 weeks (30 days) of transfer in rooting media. Data are in the form of mean±SEM, and means followed by the same letters within the columns are not significantly different at P ≤ 0.05 using Duncan's multiple range test.

Thus, highest response for root induction using different combinations of IAA with Kinetin, BAP and BAP+kinetin was found to be more by 1.4 and 3 folds with treatment T3 of IAA alone (@ 1 mg L^{-1}) for NOR and RL, respectively, as compared to control (T0) (Table 2). Increase in length of root may be due to increase in root meristematic activity and cell elongation. Previous reports also supported our findings [24,38,39].

Conclusion

The results obtained from the present study indicate that *in vitro* micropropagation of stevia in half-strength MS media supplemented with Kn (0.2 mg L^{-1}) showed early bud break and significantly increased number of shoot, shoot length (cm) and number of leaves, while overall, best response for rooting was observed in nutrient

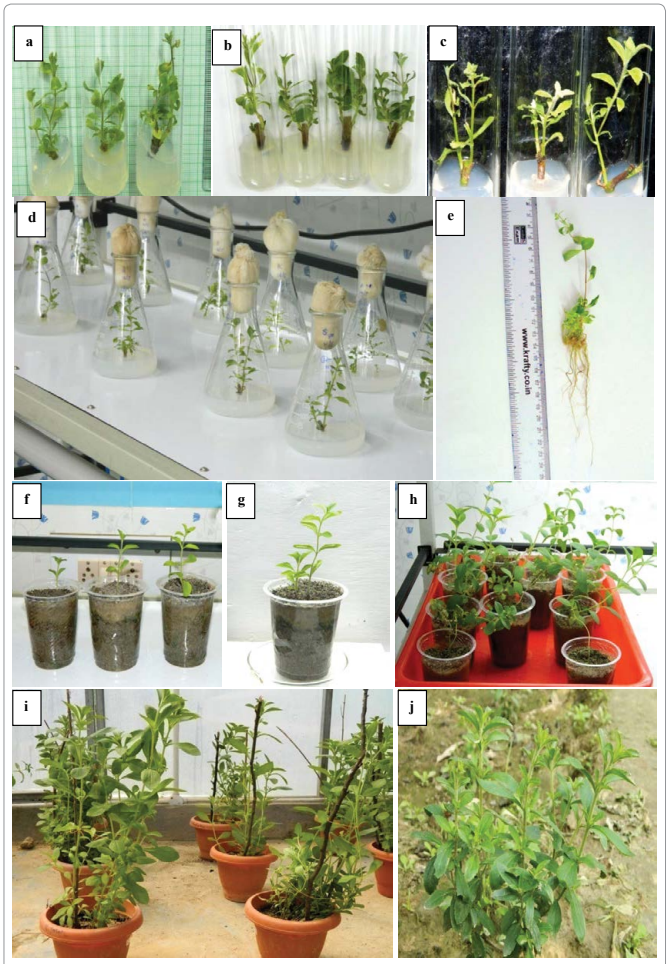

Figure 5: Shoot induction and plantlets regeneration in *Stevia rebaudiana* in half-strength MS media. (a) Regenerated shoots from nodal segment after 6 weeks of culture containing 0.2 mg L^{-1} kinetin. (b) 0.2 mg L^{-1} BAP. (c) 0.2 mg L^{-1} BAP+kinetin. (d) Shoot induction and elongation from nodal segment. (e) Rooting of the elongated shoot after 8 eeks of culture in half-strength MS medium containing both 2.0 mg L^{-1} IBA and 0.5 mg L^{-1} kinetin. (f, g, h) Acclimatized plants of *Stevia rebaudiana* in soil: perlite (2:1) after 8 weeks under control condition. (i) Fully developed *S. rebaudiana* plants grown in plastic pot. (j) Well adopted and acclimatized field grown *in vitro* raised plants [39].

Table 2: Comparative effect of IAA, Kinetin, BAP and their combinations on *in vitro* root induction in *Stevia rebaudiana*.

Treatments	Plant growth regulator (mgl^{-1})				Number of roots	Length of root (cm)
T0	IAA	0			4.50 ± 0.29^{c}	2.08 ± 0.15^{e}
T1		3			3.50 ± 0.29^{d}	3.18 ± 0.23^{d}
T2		2			5.50 ± 0.29^{b}	5.10 ± 0.04^{b}
T3		1			6.50 ± 0.29^{a}	6.00 ± 0.07^{a}
T4		0.5			5.50 ± 0.29^{b}	5.13 ± 0.06^{b}
T5		0.2			4.50 ± 0.29^{c}	4.00 ± 0.15^{c}
T0	IAA	0	Kinetin	0	4.50 ± 0.29^{b}	2.08 ± 0.15^{c}
T1		3		0.2	4.25 ± 0.25^{bc}	3.28 ± 0.18^{b}
T2		2		0.5	7.50 ± 0.29^{a}	5.58 ± 0.11^{a}
T3		1		1	6.75 ± 0.25^{a}	5.30 ± 0.14^{a}
T4		0.5		2	4.25 ± 0.25^{bc}	3.28 ± 0.11^{b}
T5		0.2		3	3.50 ± 0.29^{c}	2.20 ± 0.09^{c}
T0	IAA	0	BAP	0	4.50 ± 0.29^{c}	2.08 ± 0.15^{d}
T1		3		0.2	4.00 ± 0.00^{cd}	3.13 ± 0.12^{c}
T2		2		0.5	5.50 ± 0.29^{b}	5.15 ± 0.06^{b}
T3		1		1	6.50 ± 0.29^{a}	5.00 ± 0.23^{b}
T4		0.5		2	4.50 ± 0.29^{c}	5.23 ± 0.19^{a}
T5		0.2		3	3.50 ± 0.29^{d}	2.15 ± 0.06^{d}
T0	IAA	0	BAP+Kinetin	0	4.50 ± 0.29^{bc}	2.08 ± 0.15^{c}
T1		3		0.2	3.50 ± 0.29^{c}	2.50 ± 0.17^{bc}
T2		2		0.5	6.00 ± 0.58^{a}	5.38 ± 0.13^{a}
T3		1		1	5.50 ± 0.29^{ab}	5.35 ± 0.09^{a}
T4		0.5		2	3.50 ± 0.29^{c}	2.70 ± 0.13^{b}
T5		0.2		3	3.50 ± 0.29^{c}	2.08 ± 0.15^{c}

media supplemented with combined effect of IBA (2.0 mg L^{-1}) and Kn (0.5 mg L^{-1}). This economic micropropagation protocol can thus be used for raising stevia plants *in vitro* on large scale, making stevia more profitable commercially.

Acknowledgements

The first author is thankful to the University Grants Commission (UGC), New Delhi for providing UGC-Research Fellowship.

References

1. Das A, Gantait S, Mandal N (2011) Micropropagation of an elite medicinal plant: Stevia rebaudiana Bert Intl J Agric Res 6: 40-48.
2. Jeppesen PB, Gregersen S, Rolfsen SE, Jepsen M, Colombo M, et al. (2003) Antihyperglycemic and blood pressure-reducing effects of stevioside in the diabetic goto-kakizaki rat. Metabolism 52: 372-378.
3. Pol J, Varadova-Ostra E, Karasek P, Roth M, Benesova K, et al. (2007) Comparison of two different solvents employed for pressurised fluid extraction of stevioside from stevia rebaudiana: methanol versus water. Anal Bioanal Chem 388: 1847-1857.
4. Singh SD, Rao GP (2005) Stevia: the herbal sugar of the 21st century. Sugar Tech 7: 17-24.
5. Chan P, Xu DY, Liu JC, Chen YJ, Tomlinson B, et al. (1998) The effect of stevioside on blood pressure and plasma catecholamines in spontaneously hypertensive rats. Life sciences 63: 1679-1684.
6. Jeppesen PB, Gregersen S, Alstrup KK (2002) Stevioside induces antihyperglycemic, insulinotropic and glucagonostatic effects *in vivo*: Studies in diabetic goto-kakizaki (GK) Rats. Phytomedicine 9: 9-14.
7. Soliman HIA, Metwali EMR, Almaghrabi OA (2014) Micropropagation of *stevia rebaudiana* betroni and assessment of genetic stability of *in vitro* regenerated plants using inter simple sequence repeat (ISSR) marker. Plant Biotech 31: 249-256.
8. Alhady MRAA (2011) Micropropagation of *stevia rebaudiana* bertoni- A new sweetening crop in egypt. Global J Biotechnol Biochem 6: 178-182.
9. Geuns JMC (2003) Molecules of interest: Stevioside. Phytochem 64: 913-921.F
10. Felippe GM, Lucas NMC (1971) Estudo da viabilidade dos fructos de stevia rebaudiana bert hoehnea 1: 95-105.
11. Toffler F, Orio OA (1981) Acceni sulla pin ata tropicale 'kaa-he-e' ou 'erba dolce'. Rev Soc Sci Aliment 4: 225-230.
12. Jagatheeswari D, Ranganathan P (2012) Studies on micropropagation of *stevia rebaudiana* bert. Intl J Pharm Biol Archit 3: 315-320.
13. Naik SK, Pattnaik S, Chand PK (2000) High frequency axillary shoot proliferation and plant regeneration from cotyledonary nodes of pomegranate (punica granatum L.). Scientia Hort 85: 261-270.
14. Pande SS, Gupta P (2013) Plant tissue culture of *stevia rebaudiana* (bertoni): A Review. J Pharmacog Phytother 5: 26-33.
15. Razak UNAA, Ong CB, Yu TS, Lau LK (2014) *In vitro* micropropagation of *stevia rebaudiana* bertoni in malaysia. Brazilian Archives Biol Tech 57: 23-28.
16. Singh P, Dwivedi P, Atri N (2012) *In vitro* shoot regeneration of *Stevia rebaudiana* through callus and nodal segments. Intl J Agric Env Biotech 5: 101-108.
17. Anbazhagan M, Kalpana M, Rajendran R, Natarajan V, Dhanavel D (2010) *In vitro* production of *stevia rebaudiana* bertoni. Emirates J Food Agric 22: 216-222.
18. Mitra A, Pal A (2007) *In vitro* regeneration of *stevia rebaudiana* (Bert) from the nodal explant. J Plant Biochem Biotech 16: 59-62.
19. Sairkar P, Chandravanshi MK, Shukla NP, Mehrotra NN (2009) Mass production of an economically important medicinal plant *stevia rebaudiana* using *in vitro* propagation techniques. J Med Plant Res 3: 266-270.
20. Thiyagarajan M, Venkatachalam P (2012) Large scale *in vitro* propagation of *stevia rebaudiana* bert. for commercial application: pharmaceutically important and antidiabetic medicinal herb. Ind Crops Prod 37: 111-117.
21. Ali A, Gull I, Naz S, Afghan S (2010) Biochemical Investigation during different stages of *in vitro* propagation of *stevia rebaudiana*. Pakistan Journal of Botany 42: 2827-2837.
22. Salim MU, Shaheed MHC, Mouoztaba MMK, Belal MU, Ahmed R, et al. (2006) *In vitro* propagation of *stevia rebaudiana* bert. in bangladesh. African J Biotech 5: 1238-1240.
23. Singh P, Dwivedi P (2013) Efficient micropropagation protocols of regeneration of *stevia rebaudiana* bertoni, an anti-diabetic herb. Vegetos 26: 318-323.
24. Singh P, Dwivedi P (2014) Two-stage culture procedure using thidiazuron for efficient micropropagation of *stevia rebaudiana*, an anti-diabetic medicinal herb. Biotech 4: 431-437.
25. Duncan BD (1955) Multiple range and multiple F-test. Biometrics 11: 1-42.
26. Purohit SD, Dave A, Kukda G (1994) Micropropagation of safed musli (chlorophytum borivilianum), a rare indian medicinal herb. Plant Cell Tissue Organ Culture 39: 93-96.
27. Pattnaik S, Chand PK (1996) In vitro propagation of the medicinal herbs ocimum americanum L. syn. O. canum sims.(hoary basil) and ocimum sanctum L.(holy basil). Plant Cell Rep 15: 846-850.
28. Sen J, Sharma AK (1991) Micropropagation of *withania somnifera* from germinating seeds and shoot tips. Plant Cell Tissue Organ Culture 26: 71-73.
29. Komalivalli N, Rao MV (2000) *In vitro* micropropagation of *g. sylvestre* - a multipurpose medicinal plant. Plant Cell Tissue Organ Culture 61: 97-105.
30. Priyadarshini GR, Kumar A, Janifer X (2007) Micropropagation studies in *stevia rebaudiana* bertoni. Proceedings of National Symposium on Plant Biotechnology: New Frontiers. CIMAP, Lucknow, India.
31. Bhat SR, Chandel KPS, Malik SK (1995) Plant regeneration from various explants of cultivated *piper species*. Plant Cell Reports 14: 398-402.
32. Kukreja AK, Mathur AK, Zaim M (1990) Mass production of virus-free patchouli plants (*pogostemon cablin* (blanco) benth.) by *in vitro* culture. Tropical Agriculture 67: 101-104.
33. Mehta J, Sain M, Sharma DR, Gehlot P, Sharma P, et al. (2012) Micropropagation of an anti-diabetic plant - *stevia rebaudiana* bertoni (natural sweetener) in hadoti region of south-east rajasthan, india. ISCA J Biol Sci 1: 37-42.

34. Ranganathan J (2012) Studies on micropropagation of *stevia rebaudiana*. Intl J Pharmacol Biol Arch 3: 315-320.

35. Singh P, Dwivedi P, Atri N (2014) *In vitro* shoot multiplication of *stevia* and assessment of stevioside content and genetic fidelity of the regenerants. Sugar Tech 16: 430-439.

36. Shukla S, Shukla SK, Mishra SK (2009) *In Vitro* plant regeneration from seedling explants of *stereospermum personatum* DC: a medicinal tree. Trees 23: 409-413.

37. Deng ZC, Jin H, He H (2015) An efficient micropropagation system for *morinda officinalis* how (rubiaceae), an endangered medicinal plant. J Agr Sci Tech 17: 1609-1618.

38. Bayoudh C, Labidi R, Majdoub A, Mars M (2015) *In vitro* propagation of caprifig and female fig varieties (*ficus carica* L.) from shoot-tips. J Agr Sci Tech 17: 1597-1608.

39. Boro A, Dwivedi P, Kalita MC (2007) Micropropagation protocol of *oroxylum indicum* vent-a potent source of anti-cancerous drug. Proceedings of National symposium on plant biotechnology: new frontiers. CIMAP, Lucknow, India.

Author Affiliations

Top

Laboratory of Plant Tissue Culture and Stress Physiology, Department of Plant Physiology, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi, India