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MICRO-PROPAGATION OF *STEVIA REBAUDIANA* BERTONI THROUGH ROOT EXPLANTS

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Abstract

Protocol for *In vitro* micro-propagation of exotic medicinal plant *Stevia rubaudiana. Bert (Asteraceae)* through induction of shoots on root explants and further proliferation of multiple shoots and induction of roots on the micro shoots established. Regeneration of cultures *In vitro* was markedly influenced by the sterilant; cytokinins, auxins supplied to Murashige and Skoog's medium. Synergistic combination of 6-Benzyl aminopurine (BAP) and Thidiazuron (TDZ) evoked the regeneration of shoots with optimum numbers and maximum lengths. Subculturing of root explants on basal medium with the same concentration of BAP and TDZ resulted in the proliferation of shoots. Roots induction and maximum percentage of number of roots regeneration on Murashige & Skoog (MS) medium supplemented with 0.1mg/L IBA was observed. The *In vitro* regenerated plants were established in sand, loamy soil and farmyard manure 1:1:1. The exotic plants grow normally in the field and acclimatized in the local habitat of Peshawar valley of Pakistan.

Introduction

The medicinal plants are worthful source of many pharmaceutical products. The principal means of propagation is the conventional propagation which takes time to multiply. Due to increase in demand for the crude drugs, the plants are being over exposed (Haidar *et al*., 2012), ominous the survival of many endangered species (Sher *et al*., 2012) Also, due to fact agriculture growth, urbanization, deforestation and indiscriminate collection many valuable medicinal plant species are vanishing at an alarming rate (Ahmed *et al*., 2012) and introduction of exotic species is in a dream to become reality. Therefore, there is a need to use for advanced novel methods of culturing plant to furnish new means for conserving, quickly propagating of endangered species and also introducing exotic plants. The production of high quality planting material of exotic nature propagated from vegetative parts through tissue culture has created new opportunities in global trading. The exotic plants are beneficial for growers, farmers, nursery owners and rural employment. As exotic plants are restricted to their natural environment, the main advantage of tissue culture technology lies on production of high quality and uniform planting material that can be multiplied on a year round basis. The plant selected for such purpose is *Stevia rabuadiana* Bertoni.

Stevia rabuadiana belongs to the family Asteraceae, tribe Eupatorieae. *Stevia rabuadiana* is originally a South American wild plant (Katayama *et al*., 1976). Moises Santiago Bertoni was the first Botanist who described the plant and its sweet taste (Bertoni & Moises.1905). While Bridel & Lavielle (1931) reported the Glycosides, Stevioside and Rebaudioside that give Stevia its sweet taste. Stevioside has very high sweetening potency. It is 300 times more sweetener than cane sugar. It is used as sweetener in food products and soft drinks in Japan. Stevia is used as 40% of market sweetener in the world (Melis, 1997).

Stevia is also used as medicinal for treating heart burn and other ailments. It is recommended in treating obesity, high blood pressure and dental caries (Fajita & Edahio, 1979). Moreover, not only it has negligible effect on blood glucose but it also enhances glucose tolerance (Curi *et al.,* 1986). Being antihyperglycemic agent it stimulates the secretion of insulin from pancreas (Lu, 1993; Tomita, 1997).Therefore, it is attractive natural sweetener to diabetic patients and others who are conscious about carbohydrate controlled diets (Gegerson *et al.*, 1972). Stevioside is not only diuretic but it also possesses the antibacterial properties and dispensed in treating wounds, sores and gum diseases. Bactericidal activities of Stevia are reported by Tomita and Ghauri *et al*., (Tomita, 1997; Ghuari *et al.*, 2009). Germplasm of Stevia exhibits very poor germination. Moreover, population raised from seeds resulted in variability in the major characteristic of Stevia i.e., Stevioside and its percentage (Debnath *et al*., 2006). Vegetative propagation is very low in number of individuals that can be obtained from single plant (Yang *et al*., 1981). Keeping these difficulties in mind; tissue culture is the only technique through which one can obtain mass propagation of Stevia plants with homogeneous population. Protocols for propagation of sweet herb from leaf, nodal and axillary shoot explants are established (Yang *et al*., 1981; Lu, 1993). Ghauri *et al.,* (2009) reported the micro propagation from apical meristem and nodal segment. The present studies reveal the micro propagation of *Stevia rabaudiana* from root explants. The objective of these studies is to regenerate the non-caloric sweetener plant from root segment and to acclimatize the exotic plant in Peshawar valley (Pakistan).

Materials and Methods

Roots were excised from exotic plant. These were surface sterilized in commercial sodium hypochlorite solution 1% active chlorine and rinsed three times for 10 minutes in sterile distilled water. Root explants 1.0 cm in length were finally surface sterilized with mercuric chloride ($HgCl_2$) solution under aseptic condition for 1 minute and then washed three times with sterilized distilled water. Root explants were cultured on Murashige and Skoogs medium (Murashige & Skoog, 1962) supplemented with 2% (w/v) sucrose.

Different growth regulators i.e., Indole-butyric acid (IBA) and TDZ at different concentrations either individually or in combined form were blended with basal medium to observe their effects on shoot induction and shoot proliferation. Subeculturing to fresh media was done after four weeks. The shoots were transferred to root induction medium i.e., MS medium administered with IBA. The pH of the medium was adjusted to 5.8 with 1N NaOH and HCL and autoclaved at 15 psi and 121^{o}C for 20 minutes. Explants were oriented in the vertical position in the culture media. The cultures were incubated at temperature 25 ± 2^{o}C under 16/8- h (light/ dark) photoperiod with light supplied by fluorescent tubes at 2000 Lux.

The experiments were set up in completely randomized design. There were 10 replicates for each treatment, and experiment was repeated twice. Data of frequency of morphogenic response was recorded periodically i.e., after 4 weeks.

Results

Different concentrations of $HgCl_2$ were tested for sterilization of root explants (Table 1) 0.25% of $HgCl_2$ markedly reduced the level of contamination and mortality of explants to 100%.

Table 1. Treatment of explants with different concentration of sterilant $HgCl_2$.

Percent sterilant used	Contamination	Mortality	Survival
0.12 T_1	20.5a ± 0.7071	0.00d ± 0.0	79.5b ± 0.7071
0.25 T_2	0.00b ± 0	0.00d ± 0.0	100.0a ± 0.0
0.50 T_3	0.00b ± 0	30.5c ± 0.7071	69.5c ± 0.7071
1.00 T_4	0.00b ± 0	50.5b ± 0.7071	49.5d ± 0.7071
2.00 T_5	0.00b ± 0	70.5a ± 0.7071	29.5e ± 0.7071

Values are means ± S.D
LSD value at p≤0.5 for Contamination = 0.8129
LSD value at p≤0.5 for Mortality = 1.408
LSD value at p≤0.5 for Survival = 1.6258

Shoot induction: Root explants were cultured on MS medium supplemented with various concentrations of BAP or TDZ individually or BAP in combination with TDZ for shoot induction. The different concentrations of growth regulator BAP were tested. BAP at the concentration of 1.25mg/L exhibited the most promising results (Table 2, Fig. 1). 59.5 % of shoot induction took place on the explants nurtured on the said concentration of BAP within four weeks of culturing with an average of 3.05 cm of shoot length. Higher concentrations and the concentrations lower than 1.25mg/L of BAP reduced the shoot numbers as well as shoot length.

TDZ alone also induced shoot on explants (Table 3) but level of shoots formation was lowered i.e., 49.5% at 1mg/L. as compared to induction of shoots on BAP (59.5% at 1.5mg/L). Moreover, shoot length (2.75cm) was also less than the shoot length acquired (3.05cm) by explants implanted on MS-mixed with BAP (1.25mg/L).Explants showed shoot induction on high concentration of BAP / TDZ; however, these levels of hormones failed to proliferate further the shoot growth.

Table 2. Effect of different concentrations of BAP on shoot induction from leaf explants cultured on MS medium for 4 weeks.

BAP	% of shoot induction	Shoot length
0.12	0.0^{f} ± 0	0.0^{f} ± 0
0.25	0.0^{f} ± 0	0.0^{f} ± 0
0.50	10.5^{e} ± 0.7071	1.05^{e} ± 0.0707
0.75	19.5^{d} ± 0.7071	1.75^{d} ± 0.0707
1.00	40.5^{c} ± 0.7071	2.45^{c} ± 0.0707
1.25	59.5^{a} ± 0.7071	3.05^{a} 0.0707
1.50	50.5^{b} ± 0.7071	2.85^{b} 0.0707

Values are means + S.D ANOVA completely randomized 5 % level of significant by Duncan multiple range test

Synergistic effect of cytokinins, BAP and TDZ was then tested for shoot induction. The combined effect of varied concentrations of the said cytokinins resulted in the shoot induction. A combination of BAP (1.25mg/L) and TDZ (0.5mg/L) showed maximum (89.5a±0.707%) shoot induction efficacy with shoot length 3.75a±0.707cm.

Other combinations of BAP and TDZ (1.0mg/L +1.0mg/L) exhibited 3.75a±0.707cm shoot induction and of average 3.25a±0.707 cm shoot length (Table 4, Fig. 2).

Shoot proliferation: Multiple shoots were proliferated when the shoot cultures were sub-cultured on basal medium fortified with different concentrations and combination of BAP and TDZ.A, significantly higher number of shoots per culture was obtained on basal medium blended with BAP 1.25 mg/Land TDZ 0.5mg/L (Fig. 2). Other combination of BAP with TDZ also resulted in proliferation of multiple shoot on shoot culture, but the number of multiple shoot produced was lower than the shoots proliferated on basal medium mixed with BAP 1.25 mg/L and TDZ 0.5mg/L. (Table 4, Fig. 3).

Table 3. Effect of different concentrations of TDZ on *In vitro* shoot induction on root explants cultured on MS medium for four weeks.

TDZ (mgL^{-1})	% of explants showing shoot induction	Shoot length (cm)
0.125	20.5d ± 0.7071	0.95f ± 0.7077
0.25	30.5c ± 0.7071	1.25e ± 0.7077
0.50	40.5b ± 0.7071	1.45d ± 0.7077
0.75	50.0a ± 0.0	2.75a ± 0.7077
1.00	39.5b ± 0.7071	2.55b ± 0.7077
01.25	40.5b ± 0.7071	2.45b ± 0.7077
1.50	30.5b ± 0.7071	1.95c ± 0.7077

Values are means ± S.D
LSD value at p≤0.5 for % of explants showing shoot induction = 1.4131
LSD value at p≤0.5 for shoot length = 0.16720

Table 4. Effect of different concentrations of BAP and TDZ on shoot induction, multiplication on leaf explants cultured on MS medium for 4 weeks.

BAP + TDZ	Shoot induction	Shoot length	Multiplication
1.0 + 0.5	$64.5^{c} \pm 0.707$	$3.05^{c} \pm 0.0707$	31^{c}
1.0 + 1.0	$70.5^{b} \pm 0.707$	$3.25^{b} \pm 0.0707$	35^{b}
1.25 + 0.5	$89.5^{a} \pm 0.707$	$3.75^{a} \pm 0.0707$	40^{a}
1.25 + 1.0	$60.5^{d} \pm 0.707$	$2.85^{d} \pm 0.0707$	29^{d}
0.50 + 2.0	$45.5^{e} \pm 0.707$	$1.55^{e} \pm 0.0707$	20^{e}

S.D + LSD (mean values) for shoot induction and shoot length under different combination of BAP + Kinetin (mg/L)

Root regeneration: Regenerated shoots were excised and implanted on full MS-medium without growth regulators. No sign of root induction was noticed after 4- weeks of culturing. The regenerated shoots were then placed in 1/2 strength MS-medium mixed with varied concentrations of IBA (Table 5).

Table 5. Effect of different concentrations of IBA on *In vitro* % of shoots rooted and roots per shoot cultured on MS medium for four weeks.

IBA(mg/l)	% Shoot rooted	Roots/ shoot
0	0.0d ± 0	0.0c ± 0
0.1	95.5a ± 0.707	6.50a ± 0.707
0.2	70.5b ± 0.707	3.5 ± 0.707
0.3	39.5d ± 0.707	1.50 ± 0.707

Values are means ± S.D
LSD value at $p<0.05$ for % of explants shoot rooted =1.7002
LSD value at $p<0.05$ for roots per shoots per shoot = 1.7002

The data revealed that 95-5a±0.707 % of shoots produced roots on medium containing IBA 0.1 mg/L after 4-weeks of culturing. Moreover, 6.50 a±0.707 % roots/shoot regenerated (Fig. 4).The percentage of shoots forming roots and no. of roots produced by shoots *In vitro* depended upon the concentrations of the auxin administered. Percentage of shoots rooted and roots / shoot declined with the rise in concentration of IBA.

Acclimatization: The *In vitro* regenerated plantlets were separated from the medium. The roots were partially brown. The plant material was washed carefully with water in order to remove sticky medium. Care was taken not to place the material directly under the tap water flow. The direct water can damage the root system of juvenile plants. The rooted plants free of agar were shifted to plastic tubes having mouth with 2.5 cm diameter. The tubes were filled 3/4 with autoclaved sterilized loamy soil, sand and FYM 1:1:1 treated with agrason (0.1%) fungicide. The tubes with plants were enveloped with polythene bags. Tap water was administered daily and 1/4 strength MS solution was administered twice a week. 70 to 90% relative humidity was maintained by spraying water.

After 2 weeks the polythene bags were partially removed for 4-6 hours daily from tubes and tubes were kept inside the room and relative humidity was also reduced gradually. After 3 weeks the bags were completely removed and placed near the window inside room. The plants were transferred to the pots filled with loamy soil, sand and FYM after two weeks. The pots were placed under shade in net house (Fig. 5). From there they were transplanted to the field (Fig. 6). For this purpose pits (1x1 Sq ft in diameter and 1ft in depth) were made and filled with 1:1:1 loamy soil, sand and FYM. Plants were watered once a day. It is calculated that one explant provided average 40 numbers of shoots. Each of which produces up to 150 shoots in 3 months and total production of an average of 6000 plantlets/explants. Survival rate of plants during hardening was 70%. The protocol described here for the micro propagation of S*tevia rebaudiana* through roots explants facilitated the rapid propagation of the valuable exotic plant.

Discussion

The effect of different cytokinins, like BAP, TDZ on adventitious shoot regeneration was examined by incorporating the cytokinin alone or in combined form. Shoots induction took place at all the concentrations of BAP except the low level like 0.25 mg/L. 59.5% shoot regeneration resulted on root explants implanted on MS-medium administered with BAP (1. 25mg/L) Kelkar & Krishnamurti (1989) also reported the high frequency regeneration of shoot initiation on root explants of *Piper colurinum* cultured on MS medium blended with BAP. Silva *et al.,* (2011) also documented the shoot induction on root explants of wild passion fruit species and three commercial populations on MS medium administered with BAP. Our results are also in agreement with the findings of Yang *et al.,* (1981). They regenerated shoots on nodal segments of Stevia cultured on MS medium supplemented with BAP. The higher concentrations of BAP resulted depletion in shoot regeneration. These results are also in accordance with the findings of Sujatha & Sailaja (2005) who reported such decrease in shoot induction in Castor cultures. Thidiazuron (TDZ) cytokinin can also be used to compose a novel improved protocol for regeneration of shoots (Ghuari *et al.*, 2009). TDZ 1.0mg/L induced shoots in root explants of *Stevia rebaudiana*. Our results are in agreement with Magiols *et al.,* (1998). They reported shoot regeneration in egg plants cultured on basal medium fortified with TDZ. But level of shoot regeneration was lowered than the shoots induced by BAP. BAP regenerated more shoots 59.59±0.707 at the concentration of 1.25 mg/L

Our results also confirmed the findings of Santaram & Astarita (2003) that the addition of TDZ to medium resulted in the lower organogenic frequency in *Hypericum perforatum*. Such types of findings are also observed by Bacila *et al.,* (2010) in shoot induction on nodal segments implanted on MS-medium supplemented with TDZ.

Fig. 1. Shoot induction on MS medium fortified with BAP (1.25mg/L) of culturing.

Fig. 2. Shoot induction on MS medium administered with BAP (1.25mg/Ll) and TDZ (0.5mg/L) after 4 weeks.

Fig. 3. Roots regeneration on shoots when implanted on MS medium mixed with IBA (0.1mg/L) after 4 weeks of culturing.

Fig. 4. Shoots proliferation on basal medium blended with BAP (1.25mg/L) and TDZ (0.5mg/L).

Fig. 5. Acclimatized potted plant of *In vitro* regenerated S*etivia rebaudiana*.

Fig. 6. *In vitro* regenerated plants acclimatized and planted in the filed.

Shoot induction also depends upon the interaction between plant growth regulators and their concentration. BAP in combination with TDZ resulted in the regeneration of shoots. The combination of BAP (1.25mg^{-l}) and TDZ (0.5 mg^{-l}) evoked an optimum response showing increase in 89.5 a ± 0.707% of shoot regeneration and 3.75 a ± 0.0707cm in length of shoots.

Earlier studies also revealed the effectiveness of TDZ and Butyl amino purine cytokinins on shoot induction at several concentrations (Gegerson, 1972; Lu, 1993). For some species, the combination of TDZ plus cytokinin BAP is more effective at shoot induction than either TDZ or BAP alone. Our findings about high frequency of shoot regeneration on the root explants on basal medium fortified with TDZ and BAP are in agreement with Kern & Meyer., (1986). They stimulated shoot tip proliferation of *Aacerx freemanii* using TDZ in combination of BAP. Root regeneration is a crucial step during the micropropagation of plants. IBA is preferably used for regeneration of roots *In vitro* (De Klerke *et al.*, 1997; Ramesh *et al.*, 2002; Ramesh *et al.*, 2005).The slow movement and slow degradation of IBA facilitates its localization near sites of application and thus it better functions in inducing root (Baskaran & Jayalan, 2005). Therefore, it is preferably used for regeneration of roots *In vitro*. Optimum Number of Shoots of Stevia induced roots when cultured on half strength MS medium supplied with 0.1 mg/ L of IBA. Our results are in agreement with the findings of Baskaran and Jayabalan (2000) who regenerated roots on microshoots of *Eclipta alba when* cultured on 1/2 MS medium blended with IBA.

The optimal concentration of IBA (0.1mg/L) for production of roots on the shoots was observed. The effectiveness of IBA for rooting in regenerating shoots has also been reported by a group of researchers for medicinal plant i.e., *Hemidesmus indicus* (Fracara & Echeverrigary, 2000). In Pakistan we need to promote research on such plants that are economically beneficial and has commercial potential as demonstrated by Hussain *et al*., 2011 and Ahmad *et al*., 2011 to develop black pepper and Abbasi *et al*., 2011 for *Sinapis.*

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