In Vitro Cell.Dev.Biol.—Plant (2016) 52:174–184
DOI 10.1007/s11627-016-9749-6

MICROPROPAGATION

Synergism of polyamines and plant growth regulators enhanced morphogenesis, stevioside content, and production of commercially important natural antioxidants in *Stevia rebaudiana* Bert

Shahid Akbar Khalil[1] · Nasir Kamal[2] · Muhammad Sajid[2] · Nisar Ahmad[3] · Roshan Zamir[1] · Naveed Ahmad[2] · Shahid Ali[3]

Received: 27 June 2015 / Accepted: 26 January 2016 / Published online: 22 February 2016 / Editor: Ewen Mullins
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Abstract This study was aimed to evaluate the synergistic effects of polyamines (PAs) and plant growth regulators (PGRs) on *in vitro* propagation and stevioside production in *Stevia rebaudiana* (Stevia). A large-scale *in vitro* propagation protocol was established for leaf explants on Murashige and Skoog medium (MS) supplemented with various combinations of PAs and PGRs. The synergistic combination of spermidine (Spd, 2.0 mg L^{-1}) with 2, 4-dichlorophenoxy acetic acid (2, 4-D, 1.5 mg L^{-1}) and 6-benzyleadenine (BA, 1.5 mg L^{-1}) induced maximum callogenic response (91.7%). The combination of Spd (1.0 mg L^{-1}) and BA (1.0 mg L^{-1}) was found most effective for shoot regeneration (94.4%), mean number of shoots (14. 7), and leaves per explant (88.3). However, the combination of putrescine (Put, 2.0 mg L^{-1}) and kinetin (Kn, 2.0 mg L^{-1}) promoted mean shoot length (6.6 cm). Incorporation of either Spd or Put in combination with naphthalene acetic acid (NAA) or indole butyric acid (IBA) to culture media improved root organogenesis. Vigorous plantlets having optimum roots were successfully acclimatized in soil. Chromatographic data revealed that the synergism of Spd, BA, and Kn (2.0 mg L^{-1}) enhanced stevioside content in shoots (10.20 mg/g DSB) as compared to control (3.02 mg/g DW). Furthermore, application of Put and BA (2.0 mg L^{-1}) enhanced fresh (57.5 g L^{-1} FSB) and dry shoot biomass (9.03 g L^{-1} DSB) compared to control. In contrast, the Spd and BA (2.0 mg L^{-1}) increased antioxidant activity (80.6%) as compared to control (55.3%). Combination of Spd, BA, and GA_3 (2.0 mg L^{-1}) enhanced the production of phenolic and flavonoid contents. This is the first successful report on the application of polyamines for large-scale *in vitro* propagation and accumulation of higher stevioside content, a potential step towards industrial production.

Keywords Morphogenesis · Putrescine · Stevia · Stevioside · Spermidine · Phenolics

Introduction

Stevia rebaudiana (Stevia) belongs to family Asteraceae with a long history of ethnomedicinal uses (Aman *et al.* 2013). Traditionally, the leaves of Stevia have been used for more than 1500 yr by Guarani tribes in Brazil and Paraguay to sweeten local teas and medicines (Ahmad *et al.* 2011). Stevia leaves naturally synthesize the zero caloric sweet compound known as stevioside, which is considered as an attractive sugar alternative because of its negligible effects on blood glucose. Due to its increasing applications in food, drugs, and pharmaceutical industries, Stevia is now regularly cultivated in several countries like Canada, China, Japan, Mexico, South America, UK, USA, Korea, Malaysia, Brazil, and Paraguay (Ahmad *et al.* 2011; Dey *et al.* 2013).

Traditionally, seeds and stem cuttings have been exploited for Stevia plantations (Khalil *et al.* 2014a, b). But traditional

✉ Nisar Ahmad
ahmadn@uswat.edu.pk; nisarbiotech@gmail.com

Shahid Akbar Khalil
shahidakhalil@yahoo.com

[1] Nuclear Institute for Food and Agriculture (NIFA), Peshawar 25120, Pakistan

[2] Department of Horticulture, Faculty of Crop Production Sciences, The University of Agriculture, Peshawar 25120, Pakistan

[3] Center for Biotechnology and Microbiology, University of Swat, Swat 19200, Pakistan

cultivation is subjected to variety of pests, land availability, weather, and nutrient uptake which adversely affect the active constituents of the harvested plant. In this regard, *in vitro* regeneration is very useful for production of phytochemicals and consistent biomass (Khalil *et al.* 2014a, b). Various *in vitro* and *ex vitro* culture systems including adventitious root culture, callus culture, cell culture, *in vitro* shoots, *in vitro* plantlets, acclimated plantlets, floral parts, seeds, intact leaves, and roots have been investigated for stevioside production (Bondarev *et al.* 2003; Rajasekaran *et al.* 2007, 2008; Ladygin *et al.* 2008; Giridhar *et al.* 2010; Woelwer-Rieck *et al.* 2010; Reis *et al.* 2011; Mathur and Shekhawat 2012; Aman *et al.* 2013; Dey *et al.* 2013; Khalil *et al.* 2014a, b; 2015).

Elicitation of culture media either with physical or chemical agents is one of the important strategies to improve *in vitro* culture and production of medicinally important metabolites (Ali and Abbasi 2014). The addition of polyamines (PAs) especially putrescine (Put) and spermidine (Spd) to the culture media interacts with biological molecules due to their cationic nature, and as a result, stimulates cellular multiplication and morphogenesis (Bagni and Tassoni 2001; Kevers *et al.* 2002; Rajesh *et al.* 2003; Bertoldi *et al.* 2004). Moreover, PAs act as growth regulators and hormonal messengers and enhance the production of betalaine, coumarin, esculin, esculetin, and rosmarinic acid in various medicinal species including *Cichorium intybus* (witloof chicory), *Beta vulgaris*, and *Nepeta cataria* (Bais and Ravishankar 2003; Bais *et al.* 2004; Suresh *et al.* 2004). In earlier studies, the presence of cytokinin, especially BA, in culture media, significantly influenced the morphogenetic potential and secondary metabolite production in medicinal plant species (Ahmad *et al.* 2013, 2014).

The *in vitro* production of phytochemicals in plant cell and organ cultures is an attractive alternative compared to extraction from different parts of naturally growing plants (Skrzypczak-Pietraszek *et al.* 2014). These culture systems are useful for production of valuable plant-specific metabolites such as pharmaceuticals, neutraceutical, fine chemicals, pigments, and cosmetics (Verpoorte *et al.* 2002; Kolewe *et al.* 2008). Furthermore, to produce large amounts of value added compounds, these culture systems seem to be more promising and reliable than field cultivation.

The aim of the present study was to establish a promising and cost-effective system for large-scale plant multiplication with enhanced stevioside production. Various combinations of polyamines and plant growth regulators were applied to investigate their effect on micropropagation and stevioside accumulation in shoots *in vitro*. Studies have been conducted on the composition of stevioside in Stevia leaves, but the effect of different PAs and PGRs on stevioside have not yet been reported. Unfortunately, there are some major issues regarding the cultivation of Stevia. Stevia seeds do not produce homogenous populations and vary in stevioside content. Furthermore, seed germination percentage is very low, and the seeds lose viability if stored. Lower survival percentage of stem cuttings and lack of availability of sufficient stocks of plants are some of other limiting factors in large-scale production of Stevia (Ahmad *et al.* 2011). To avoid these limiting factors, plant cell and tissue culture is the most appropriate technique for consistent production of biomass and enhanced bioactive compounds (Ahmad *et al.* 2010; 2013; 2014). This promising approach will provide an opportunity for the selection of suitable PGR and PA for micropropagation and higher stevioside production for commercial applications.

Material and Methods

Micropropagation Leaf explants were excised from 50-d-old potted plants of Stevia at Nuclear Institute for Food and Agriculture (NIFA), Peshawar in April, 2014 (Fig. 1a–b). To develop callus cultures, 3–4-mm^2 leaf pieces were placed on MS media (Murashige and Skoog 1962) containing putrescine (Put; Phytotech Labs America, Kansas, Kansas) or spermidine (Spd) in combination with 2, 4-dichlorophenoxy acetic acid (2, 4-D) and 6-benzyleadenine (BA) as indicated (Table 1). MS medium without plant growth regulators (PGRs, MS0) was used as a control. The MS medium was augmented with 30 g L^{-1} sucrose, solidified with 8 g L^{-1} agar (Agar Technical LP0013, Oxoid, Hampshire, England), the pH was adjusted to 5.6-5.8 and autoclaved at 121°C for 20 min. These cultures were maintained in a growth room at 25 ± 1°C under a 16-hr photoperiod with a light intensity of ~40–50 μmol m^{-2} s^{-1} provided by fluorescent tube lights (20 W, Toshiba FL20T9D/19; 380–780 nm, Tokyo, Japan). Each treatment was divided into three independent experiments. Each experiment was designed on completely randomized design (CRD). After 30 d of callus establishment, the average weights were recorded using each replicate as percent callus induction. The callus color and morphology were also recorded.

Fresh callus was shifted to new medium containing Put or Spd in combination with BA, kinetin (Kn), or gibberellic acid (GA_3) as indicated (Table 2) for shoot morphogenesis. Data on percent shooting response, mean shoot length, average number of shoots and leaves per explant was collected after 30 d.

For root organogenesis, shoots of a suitable length were transferred to fresh medium incorporated with Put or Spd in combination with IBA, IAA, or NAA as indicated (Table 3). Data on percent rooting, mean root length, and average number of roots per plantlet was recorded after 35 d of shoot culture. Plantlets having optimum roots were gradually shifted to a combination of soil, sand, and clay (2:1:1). Completely established plantlets were obtained after 2 mo of their transfer to soil.

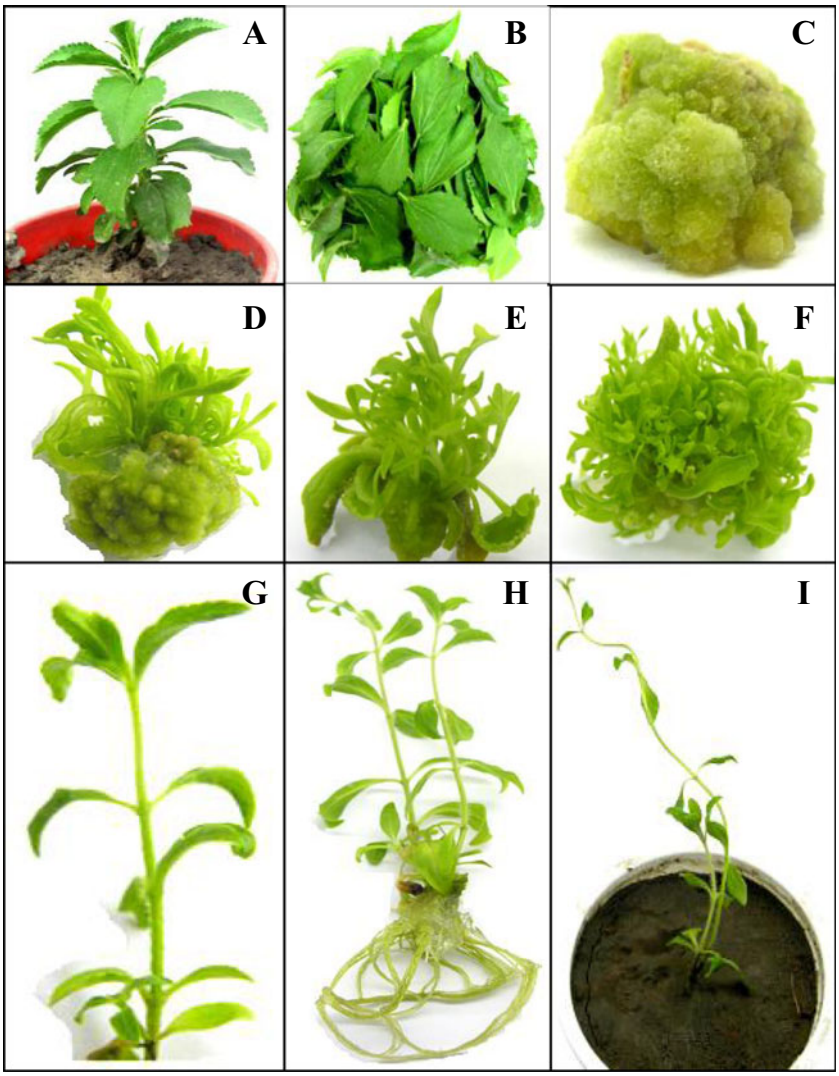

Figure 1. Regeneration of Stevia from leaf explants, (*A*) mother plant, (*B*) leaf explants, (*C*) callus, (*D*) shoots regeneration (*E* and *F*) shoot multiplication (*G*) shoot elongation (*H*) rooting and (*I*) acclimatization.

Fresh and dry biomass determination Fresh shoots were collected from cultured flasks after 30 d of application of PAs and various cytokinins. For determination of fresh shoot biomass (FSB), solid media and surface water were carefully removed by using autoclaved filter paper (Whatman Ltd., Kent, England) and weighed as gram/6-flasks. Similarly, for determination of dry shoot biomass (DSB), shoots were dried in an oven at 60°C for 24 hrs, weighed and expressed as gram/6-flasks.

Quantification of stevioside content The dried shoots from each treatment were ground in mortar and pestle to a fine powder. The stevioside content in each of the treated samples was determined following Aman *et al.* (2013) and Dey *et al.* (2013). The extracts were prepared in analytical grade ethanol by dissolving exactly 20 mg of dried shoot powder in 10 ml of ethanol. HPLC was performed using Shimadzu system (LC-8A; Kyoto, Japan) for stevioside quantification using a C-18 column (150 × 4.6 mm), variable wavelength detector, binary pump, solvent vacuum degasser, and 10-µl injection loop. Analytical grade methanol (70 % (*v/v*); A) and water (30 % (*v/v*); B) were used as mobile phase with a flow rate of 1.5 ml min^{-1}. Stevioside standard (Sigma; St. Louis, Missouri) was prepared in HPLC grade water (200 µg ml^{-1}), and the retention of the extract was compared with the standard retention time. The results obtained for each sample were expressed in mg/g of dry weight (DW).

Determination of phenolic and flavonoid contents For extract preparation, the dried shoots were ground using a mortar and pestle. Total phenolic content (TPC) was determined according to the protocol of Velioglu *et al.* (1998). The total flavonoid content (TFC) was determined according to the method of Chang *et al.* (2002). Phenols and flavonoid were dissolved by extracting 20-mg leaf powder in 10 ml ethanol. Total phenol was determined by mixing 0.1 ml of Folin-Ciocalteus reagent with extract (0.03 ml) and distilled water (2.5 ml), centrifuged (10,000 rpm, 14 min) and incubated for

Table 1. The effect of growth regulators and polyamines on callus induction in Stevia

Treatments	MS + PGRs (mg L^{-1})	Mean % callus induction (±S.E.)	Callus color	Callus morphology
T1	2, 4-D (0.5) + Put (0.5)	33.3 ± 1.7*e*	Yellow	Granular
T2	2, 4-D (1.0) + Put (1.0)	33.3 ± 1.6*e*	Green	Spongy
T3	2, 4-D (1.5) + Put (1.5)	50.0 ± 2.5*d*	Green	Granular and spongy
T4	2, 4-D (2.0) + Put (2.0)	54.2 ± 2.7*d*	Yellow	Spongy
T5	2, 4-D (0.5) + Spd (0.5)	29.2 ± 1.5*f*	Green	Spongy
T6	2, 4-D (1.0) + Spd (1.0)	41.7 ± 2.1*de*	Yellow green	Spongy
T7	2, 4-D (1.5) + Spd (1.5)	45.8 ± 2.3*de*	Yellow green	Spongy
T8	2, 4-D (2.0) + Spd (2.0)	70.8 ± 3.5*bc*	Yellow	Granular and spongy
T9	2, 4-D (0.5) + BA (0.5) + Put (0.5)	66.7 ± 3.3*c*	Green	Friable and granular
T10	2, 4-D (1.0) + BA (1.0) + Put (1.0)	75.0 ± 3.8*b*	Green yellow	Granular
T11	2, 4-D (1.5) + BA (1.5) + Put (1.5)	50.0 ± 2.5*d*	Green	Granular
T12	2, 4-D (2.0) + BA (2.0) + Put (2.0)	45.8 ± 2.3*de*	Green	Friable clump
T13	2, 4-D (0.5) + BA (0.5) + Spd (0.5)	29.2 ± 1.5*f*	Yellow green	Clump and granular
T14	2, 4-D (1.0) + BA (1.0) + Spd (1.0)	83.3 ± 4.2*ab*	Yellow green	Granular
T15	2, 4-D (1.5) + BA (1.5) + Spd (1.5)	87.5 ± 4.4*a*	Green	Friable and granular
T16	2, 4-D (2.0) + BA (2.0) + Spd (2.0)	91.7 ± 4.6*a*	Green	Friable and granular
Control	MS0	0	NA	NA

Percent callus induction, callus color and morphological observations on medium containing *T1* (0.5 mg L^{-1} 2, 4-D and Put); *T2* (1.0 mg L^{-1} 2, 4-D and Put); *T3* (1.5 mg L^{-1} 2, 4-D and Put); *T4* (2.0 mg L^{-1} 2, 4-D and Put); *T5* (0.5 mg L^{-1} 2, 4-D and Spd); *T6* (1.0 mg L^{-1} 2, 4-D and Spd); *T7* (1.5 mg L^{-1} 2, 4-D and Spd); *T8* (2.0 mg L^{-1} 2, 4-D and Spd); *T9* (0.5 mg L^{-1} 2, 4-D, BA and Put); *T10* (1.0 mg L^{-1} 2, 4-D, BA and Put); *T11* (1.5 mg L^{-1} 2, 4-D, BA and Put); *T12* (2.0 mg L^{-1} 2, 4-D, BA and Put); *T13* (0.5 mg L^{-1} 2, 4-D, BA and Spd); *T14* (1.0 mg L^{-1} 2, 4-D, BA and Spd); *T15* (1.5 mg L^{-1} 2, 4-D, BA and Spd); *T16* (2.0 mg L^{-1} 2, 4-D, BA and Spd) and *T17* (MS0). Mean ± SE was collected from three independent experiments. Mean values with the same *letter* were significantly different at $P < 0.05$ according to Tukey's test

Table 2. The effect of growth regulators and polyamines on shoot organogenesis in Stevia

Treatments	MS + PGRs (mg L^{-1})	Mean % shoot induction (±S.E.)	Mean shoot length (cm) (±S.E.)	Mean shoots/explant (±S.E.)	Mean leaves/explant (±S.E.)
T1	BA (1.0) + Put (1.0)	83.6 ± 4.2*b*	3.9 ± 0.2*b*	6.3 ± 0.3*d*	70.0 ± 3.5*bc*
T2	BA (2.0) + Put (2.0)	91.7 ± 4.5*a*	4.1 ± 0.2*b*	4.7 ± 0.2*d*	51.3 ± 2.6*de*
T3	Kn (1.0) + Put (1.0)	55.5 ± 2.8*e*	4.8 ± 0.3*b*	4.7 ± 0.2*d*	51.3 ± 2.5*de*
T4	Kn (2.0) + Put (2.0)	72.2 ± 3.6*c*	6.6 ± 0.3*a*	4.0 ± 0.2*d*	42.3 ± 2.2*e*
T5	BA (1.0) + Spd (1.0)	94.4 ± 4.7*a*	4.7 ± 0.2*b*	14.7 ± 0.7*c*	88.3 ± 4.4*a*
T6	BA (2.0) + Spd (2.0)	66.6 ± 3.3*d*	2.3 ± 0.1*cd*	6.3 ± 0.3*d*	47.3 ± 2.4*e*
T7	Kn (1.0) + Spd (1.0)	75.0 ± 3.7*c*	4.5 ± 0.2*b*	3.7 ± 0.2*d*	21.7 ± 1.1*f*
T8	Kn (2.0) + Spd (2.0)	38.9 ± 1.9*f*	4.2 ± 0.2*b*	3.3 ± 0.2*d*	23.0 ± 1.2*f*
T9	BA (1.0) + Kn (1.0) + Put (1.0)	85.7 ± 4.3*b*	2.4 ± 0.1*c*	13.0 ± 0.6*c*	64.0 ± 3.2*cd*
T10	BA (2.0) + Kn (2.0) + Put (2.0)	77.7 ± 3.9*c*	1.7 ± 0.1*cd*	4.3 ± 0.2*d*	43.0 ± 2.2*e*
T11	BA (1.0) + GA_3 (1.0) + Put (1.0)	80.0 ± 4.1*b*	1.6 ± 0.1*cd*	5.7 ± 0.3*d*	49.0 ± 2.5*e*
T12	BA (2.0) + GA_3 (2.0) + Put (2.0)	72.2 ± 3.6*c*	1.3 ± 0.1*cd*	14.0 ± 0.7*c*	68.3 ± 3.4*bc*
T13	BA (1.0) + Kn (1.0) + Spd (1.0)	86.7 ± 4.3*b*	2.3 ± 0.1*cd*	11.7 ± 0.6*c*	68.7 ± 3.4*bc*
T14	BA (2.0) + Kn (2.0) + Spd (2.0)	88.9 ± 4.4*ab*	2.3 ± 0.1*c*	15.0 ± 0.7*c*	69.3 ± 3.5*bc*
T15	BA (1.0) + GA_3 (1.0) + Spd (1.0)	76.3 ± 3.8*c*	1.9 ± 0.1*cd*	27.7 ± 1.3*a*	80.7 ± 4.1*ab*
T16	BA (2.0) + GA_3 (2.0) + Spd (2.0)	72.2 ± 3.6*c*	1.3 ± 0.07*cd*	20.0 ± 1*b*	87.3 ± 4.4*a*
Control	MS0	8.7 ± 0.4*g*	1.1 ± 0.05*d*	1.9 ± 0.1*d*	15.7 ± 0.8*f*

Percent shoot induction, mean shoot length, mean number of shoots and leaves per explant on medium containing *T1* (1.0 mg L^{-1} BA and Put); *T2* (2.0 mg L^{-1} BA and Put); *T3* (1.0 mg L^{-1} Kn and Put); *T4* (2.0 mg L^{-1} Kn and Put); *T5* (1.0 mg L^{-1} BA and Spd); *T6* (2.0 mg L^{-1} BA and Spd); *T7* (1.0 mg L^{-1} Kn and Spd); *T8* (2.0 mg L^{-1} Kn and Spd); *T9* (1.0 mg L^{-1} BA, Kn, and Put); *T10* (2.0 mg L^{-1} BA, Kn, and Put); *T11* (1.0 mg L^{-1} BA, GA_3, and Put,); *T12* (2.0 mg L^{-1} BA, GA_3, and Put); *T13* (1.0 mg L^{-1} BA, Kn, and Spd); *T14* (2.0 mg L^{-1} BA, Kn, and Spd); *T15* (1.0 mg L^{-1} BA, GA_3, and Spd); *T16* (2.0 mg L^{-1} BA, GA_3, and Spd) and *T17* (MS0). Mean ± SE was collected from three independent experiments. Mean values with the same *letter* were significantly different at $P < 0.05$ according to Tukey's test

Table 3. The effect of growth regulators and polyamines on root organogenesis in Stevia

Treatments	MS + PGRs (mg L^{-1})	Mean root induction (%) (±S.E.)	Mean root length (cm) (±S.E.)	Mean roots/shoot (±S.E.)
T1	IBA (1.0) + Put (1.0)	71.4 ± 3.6*ab*	7.2 ± 0.4*de*	5.9 ± 0.3*i*
T2	IBA (2.0) + Put (2.0)	62.7 ± 3.2*bc*	8.1 ± 0.4 *cd*	8.4 ± 0.4*hi*
T3	NAA (1.0) + Put (1.0)	78.6 ± 3.9*a*	12.5 ± 0.6*a*	23.3 ± 1.2*c*
T4	NAA (2.0) + Put (2.0)	45.8 ± 2.3*de*	3.6 ± 0.2*g*	10.3 ± 0.5*h*
T5	IBA (1.0) + Spd (1.0)	54.2 ± 2.7 *cd*	9.1 ± 0.5*c*	15.2 ± 0.7 *g*
T6	IBA (2.0) + Spd (2.0)	75.3 ± 3.7*ab*	11.6 ± 0.6*a*	20.5 ± 1.2*de*
T7	NAA (1.0) + Spd (1.0)	80.6 ± 4.1*a*	10.3 ± 0.5*b*	19.7 ± 0.9*de*
T8	NAA (2.0) + Spd (2.0)	36.7 ± 1.2*ef*	2.1 ± 0.1*hij*	16.2 ± 0.8 *fg*
T9	NAA (1.0) + IBA (1.0) + Put (1.0)	26.7 ± 1.3*f*	6.7 ± 0.3*ef*	18.2 ± 0.9*ef*
T10	NAA (2.0) + IBA (2.0) + Put (2.0)	11.1 ± 0.6*g*	1.8 ± 0.1*ij*	9.5 ± 0.5 *h*
T11	IAA (1.0) + IBA (1.0) + Put (1.0)	46.6 ± 2.3*de*	7.7 ± 0.4*de*	30.8 ± 1.5*a*
T12	IAA (2.0) + IBA (2.0) + Put (2.0)	40.0 ± 2*def*	6.7 ± 0.3*ef*	20.5 ± 1.1*de*
T13	NAA (1.0) + IBA (1.0) + Spd (1.0)	7.0 ± 0.4*g*	2.8 ± 0.2*ghi*	22.3 ± 1.2*cd*
T14	NAA (2.0) + IBA (2.0) + Spd (2.0)	4.6 ± 0.3*g*	1.0 ± 0.1*j*	9.9 ± 0.5 *h*
T15	IAA (1.0) + IBA (1.0) + Spd (1.0)	7.3 ± 0.4*g*	5.5 ± 0.3*f*	20.4 ± 1.1*de*
T16	IAA (2.0) + IBA (2.0) + Spd (2.0)	11.0 ± 0.5*g*	3.2 ± 0.2*gh*	27.4 ± 1.4*b*
Control	MS0	5.3 ± 0.3*g*	0.9 ± 0.1*j*	2.4 ± 0.1*j*

Percent root induction, mean root length and mean number of roots per shoot on medium containing *T1* (1.0 mg L^{-1} IBA and Put); *T2* (2.0 mg L^{-1} IBA and Put); *T3* (1.0 mg L^{-1} NAA and Put); *T4* (2.0 mg L^{-1} NAA and Put); *T5* (1.0 mg L^{-1} IBA and Spd); *T6* (2.0 mg L^{-1} IBA and Spd); *T7* (1.0 mg L^{-1} NAA and Spd); *T8* (2.0 mg L^{-1} NAA and Spd); *T9* (1.0 mg L^{-1} NAA, IBA, and Put); *T10* (2.0 mg L^{-1} NAA, IBA, and Put); *T11* (1.0 mg L^{-1} IAA, IBA, and Put); *T12* (2.0 mg L^{-1} IAA, IBA, and Put); *T13* (1.0 mg L^{-1} NAA, IBA, and Spd); *T14* (2.0 mg L^{-1} NAA, IBA, and Spd); *T15* (1.0 mg L^{-1} IAA, IBA, and Spd); *T16* (2.0 mg L^{-1} IAA, IBA, and Spd) and *T17* (MS0). Mean ± SE was collected from three independent experiments. Mean values with the same *letter* were significantly different at $P < 0.05$ according to Tukey's test

30 min in the dark. After incubation, the mixture was filtered through 45-μm membrane, and the A_{760} of each sample was calibrated against standard gallic acid (0.03 ml of each 2-20 μg). Results were expressed as gallic acid mg/g DSB. For TFC, 0.25 ml extract was mixed with $AlCl_3$ (0.075 ml, 5 % *w/v*), 1.25 ml distilled water, and 0.5 ml of NaOH, centrifuged (10,000 rpm, 14 min) and incubated for 30 min. The A_{510} of each sample was calibrated against standard rutin (0.25 ml of each 2–20 μg). The total flavonoid content was expressed as rutin mg/g DSB.

Determination of antioxidant activity 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity (DRSA) in shoots was determined according to the method of Amarowicz *et al.* (2004). Methanolic extract (1.0 ml, 5-mg shoot powder/20 ml) of each sample was mixed with 2.0 ml of DPPH solution (0.3 mg/20 ml) in quadruplicate. The mixture was incubated in the dark for approximately 30 min and the A_{517} was measured at room temperature. DRSA was calculated as percentage of DPPH discoloration using the following equation: $DRSA\ (\%) = 100 \times (1 - A_P/A_D)$, where A_P represents absorbance of shoots extract at 517 nm, and A_D is the absorbance of the DPPH solution without extract.

Statistical analysis The effect of plant growth regulators and polyamines on growth and secondary metabolite production was analyzed by one-way analysis of variance (ANOVA). Values are mean of three replicates from duplicate experiments. The significance of differences between means of the treatments was analyzed by the least significant difference (LSD) at a probability level of 0.05. Figures were generated using Origin Lab (8.5) software.

Results and Discussion

Optimization of static cultures from leaf explants Callogenesis was initiated from leaf explants on MS media incorporated with various concentrations of PAs in combination with auxin or cytokinin. The application of exogenous PAs increased callogenic frequency. The addition of higher concentrations of Spd, 2, 4-D, and BA (2.0 mg L^{-1}) enhanced callus formation (91.7%) from Stevia leaf explants (Table 1, Fig. 1c). However, lower (0.5 mg L^{-1}) concentrations showed least callogenic responses. The literature is scarce regarding the effect of PAs on callogenesis in Stevia; however, the effect of various auxin/cytokinin concentrations on callus formation is widely reported (Bondarev *et al.* 2003; Sivaram and Mukundan 2003; Hwang 2006; Ibrahim *et al.* 2008;

Ladygin *et al.* 2008; Sreedhar *et al.* 2008; Jain *et al.* 2009; Reis *et al.* 2011). It has been reported that the metabolic pathway of PAs is linked with ethylene, auxin, and cytokinin which markedly influence the formation of calli (Bais and Ravishankar 2003). In contrast, Bajaj *et al.* (1970) reported that the addition of Spd to the medium inhibited callus proliferation; however, our results showed positive impact of Spd on callogenesis. The difference in data could be attributed to plants species, type of explants, and concentration of applied PGRs.

Shoot morphogenesis The applications of various concentrations of Spd and Put in combination with auxin/cytokinin promoted shoot morphogenesis (38.9 to 94.4%) in Stevia (Table 2; Fig. 1*d*). Viable calli of each treatment were shifted to shoot regeneration media, incubated, and data was collected after 6-wk culture. Higher shoot morphogenic potential (94.4%) was obtained for the medium containing combination of Spd and BA (1.0 mg L^{-1}, Fig. 1*e*). As compared to Spd, higher concentration of Put in combination with BA (2.0 mg L^{-1}) promoted shoot regeneration (91.7%) as shown in Table 2. In contrast, the lowest response (38.9%) was observed with Kn and Spd (2.0 mg L^{1}) as compared to control (8.7%). Different studies conducted on shoot morphogenesis were reviewed to compare with the current results; however, this study is the first effort to study the effect of polyamines on micropropagation of Stevia. The effect of cytokinin alone or auxin alone or different ratios of cytokinin/auxin on shoot morphogenesis in Stevia is widely reported but literature is limited regarding the effect of PAs on shoot morphogenesis (Sandal *et al.* 2001; Bondarev *et al.* 2003; Hwang 2006; Ibrahim *et al.* 2008; Ali *et al.* 2010). Shyamali and Hattori (2007) reported that the morphogenetic response to polyamines depended on hormone concentration. Thiruvengadam *et al.* (2012) recently reported that addition of PGR and Spd to the medium enhanced shoot regeneration from callus cultures of *Momordica charantia*. It was better to use a synergistic combination of PAs with other PGRs for shoot regeneration in *Lagenaria siceraria* (Shyamali and Hattori 2007). Shankar *et al.* (2011) reported that adding different PAs in the medium enhanced the shoot regeneration frequency in *Saccharum officinarum* L.

In order to enhance the number of shoots/explant, MS medium was augmented with BA, Kn, GA_3, Put, and Spd (1.0 and 2.0 mg L^{-1}) along with control (MS0) treatment. The highest number of shoots/explant (27.7) was recorded on MS media supplemented with GA_3, BA, and Spd (1.0 mg L^{-1}, Table 2, Fig. 1f). It is evident from the literature that polyamines play a major role in morphogenic processes during plant tissue culture. Martin-Tanguy (2001) suggested

Table 4. The effect of growth regulators and polyamines on fresh and dry biomass and stevioside content in Stevia shoots *in vitro*

Treatments	MS + PGRs + PAs (mg L^{-1})	Mean fresh shoots biomass (g L^{-1} FSB) (±S.E.)	Mean dry shoots biomass (g L^{-1} DSB) (±S.E.)	Steviosides content (mg/g DSB) (±S.E.)
*T*1	BA (1.0) + Put (1.0)	36.13 ± 1.12*ef*	5.672 ± 0.28*cd*	5.178 ± 0.14*bc*
*T*2	BA (2.0) + Put (2.0)	57.51 ± 3.12*a*	9.029 ± 0.45*a*	3.979 ± 0.16*e*
*T*3	Kn (1.0) + Put (1.0)	47.53 ± 0.65*c*	7.462 ± 0.37*bc*	3.008 ± 0.37*cd*
*T*4	Kn (2.0) + Put (2.0)	35.36 ± 3.74*ef*	5.551 ± 0.27*cd*	2.206 ± 0.25*d*
*T*5	BA (1.0) + Spd (1.0)	41.93 ± 0.39*d*	6.583 ± 0.32*c*	2.010 ± 0.13*d*
*T*6	BA (2.0) + Spd (2.0)	53.73 ± 4.22*b*	8.435 ± 0.42*b*	3.295 ± 0.57*cd*
*T*7	Kn (1.0) + Spd (1.0)	37.36 ± 2.74*e*	5.865 ± 0.29 *cd*	6.076 ± 0.40*b*
*T*8	Kn (2.0) + Spd (2.0)	41.92 ± 5.33*d*	6.581 ± 0.32*c*	3.040 ± 0.40*cd*
*T*9	BA (1.0) + Kn (1.0) + PUT (1.0)	49.35 ± 4.22*bc*	7.747 ± 0.38*bc*	5.041 ± 0.61*bc*
*T*10	BA (2.0) + Kn (2.0) + PUT (2.0)	37.14 ± 0.87*e*	5.830 ± 0.29*cd*	4.391 ± 0.09*c*
*T*11	BA (1.0) + GA_3 (1.0) + PUT (1.0)	41.69 ± 2.73*d*	6.545 ± 0.32*c*	3.773 ± 0.39*cd*
*T*12	BA (2.0) + GA_3 (2.0) + PUT (2.0)	33.98 ± 3.29*f*	5.334 ± 0.26*d*	3.582 ± 0.50*cd*
*T*13	BA (1.0) + Kn (1.0) + Spd (1.0)	57.67 ± 2.66*a*	9.054 ± 0.45*a*	6.190 ± 0.26*b*
*T*14	BA (2.0) + Kn (2.0) + Spd (2.0)	47.51 ± 5.52*c*	7.459 ± 0.37*bc*	10.20 ± 0.70*a*
*T*15	BA (1.0) + GA_3 (1.0) + Spd (1.0)	42.23 ± 0.92*cd*	6.630 ± 0.33*c*	3.587 ± 0.30*cd*
*T*16	BA (2.0) + GA_3 (2.0) + Spd (2.0)	42.32 ± 1.19*cd*	6.644 ± 0.33*c*	3.685 ± 0.71*cd*
*T*17	MS0	11.12 ± 2.10*g*	5.532 ± 0.27*cd*	3.015 ± 0.60*cd*

Fresh shoots biomass, dry shoots biomass and stevioside content in shoots grown on medium containing *T1* (1.0 mg L^{-1} BA and Put); *T2* (2.0 mg L^{-1} BA and Put); *T3* (1.0 mg L^{-1} Kn and Put); *T4* (2.0 mg L^{-1} Kn and Put); *T5* (1.0 mg L^{-1} BA and Spd); *T6* (2.0 mg L^{-1} BA and Spd); *T7* (1.0 mg L^{-1} Kn and Spd); *T8* (2.0 mg L^{-1} Kn and Spd); *T9* (1.0 mg L^{-1} BA, Kn, and Put); *T10* (2.0 mg L^{-1} BA, Kn, and Put); *T11* (1.0 mg L^{-1} BA, GA_3, and Put,); *T12* (2.0 mg L^{-1} BA, GA_3, and Put); *T13* (1.0 mg L^{-1} BA, Kn, and Spd); *T14* (2.0 mg L^{-1} BA, Kn, and Spd); *T15* (1.0 mg L^{-1} BA, GA_3, and Spd); *T16* (2.0 mg L^{-1} BA, GA_3, and Spd) and *T17* (MS0). Mean ± SE was collected from three independent experiments. Mean values with the same *letter* were significantly different at $P < 0.05$ according to Tukey's test

Table 5. Comparison of stevioside concentration with previous studies

Reference	Plant tissues	Stevioside (mg/g DW)
This study	*In vitro* shoots	10.20
Aman *et al.* (2013)	*In vitro* shoots	0.082
Dey *et al.* (2013)	*In vitro* microshoots	2.9
Mathur and Shekhawat (2012)	Cell suspension	0.381
Reis *et al.* (2011)	Adv. root culture	0
Woelwer-Rieck *et al.* (2010)	Intact leaves	8.75
Ladygin *et al.* (2008)	Calli cells	0.06
Ladygin *et al.* (2008)	*In vitro* shoots	0.28
Bondarev *et al.* (2003)	*In vitro* shoots	~6.23

that exogenous polyamines along with other PGRs triggered proliferation and growth of plant cells and also led to the formation of adventitious shoot. The current data is in agreement with the results of Vasudevana *et al.* (2008), which showed that a synergistic combination of PAs and PGRs promoted shoot multiplication in *Cucumis sativus* L. The results in the present study showed that combination of GA_3, BA, and Spd supported shoot multiplication in Stevia (Fig. 1*f*). Moreover, average shoot length (6.6 cm) was observed on medium incorporated with Put and Kn (2.0 mg L^{-1}, Fig. 1*g*). Maximum number (88.3) of leaves/explant was recorded on medium containing BA and Spd (1.0 mg L^{-1}), followed by treatment fortified with GA_3, BA, and Spd (2.0 mg L^{-1}) that produced 87.3 leaves per explants. The current results showed a positive correlation of PAs and PGRs on shoot morphogenesis.

Root organogenesis Vigorous shoots were transferred to fresh medium with various concentrations of auxin and PAs for the root development. All the treatments used promoted root initiation. Maximum rooting (80.6%) was observed on medium fortified with Spd and NAA (1.0 mg L^{-1}) followed by combination of Put and Spd (1.0 mg L^{-1}, Table 3, Fig. 1*h*). Earlier studies of Chotikadachanarong and Srisuluk (2013) reported that NAA is an essential component for root production. Similarly, the addition of Put and NAA (1.0 mg L^{-1}) improved mean root length (12.5 cm) as compared to other treatments and control. Moreover, combination of Spd and IBA (2.0 mg L^{-1}) also produced elongated roots (11.6 cm)

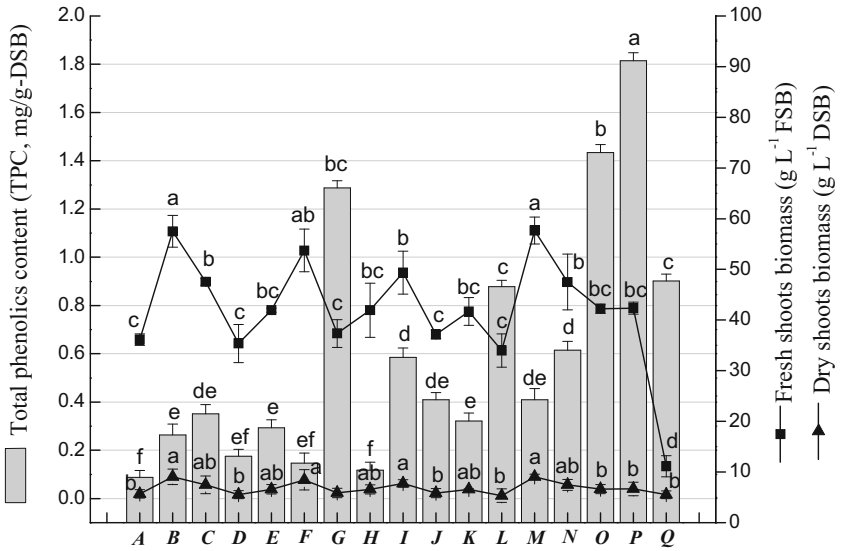

Figure 2. Total phenolics content and its correlation with fresh and dry biomass in shoots treated with *A*, Put (1.0 mg L^{-1}), BA (1.0 mg L^{-1}); *B*, Put (2.0 mg L^{-1}), BA (2.0 mg L^{-1}); *C*, Put (1.0 mg L^{-1}), Kn (1.0 mg L^{-1}); *D*, Put (2.0 mg L^{-1}), Kn (2.0 mg L^{-1}); *E*, Spd (1.0 mg L^{-1}), BA (1.0 mg L^{-1}); *F*, Spd (2.0 mg L^{-1}), BA (2.0 mg L^{-1}); *G*, Spd (1.0 mg L^{-1}), Kn (1.0 mg L^{-1}); *H*, Spd (2.0 mg L^{-1}), Kn (2.0 mg L^{-1}); *I*, Put (1.0 mg L^{-1}), BA (1.0 mg L^{-1}), Kn (1.0 mg L^{-1}); *J*, Put (2.0 mg L^{-1}), BA (2.0 mg L^{-1}), Kn (2.0 mg L^{-1}); *K*, Put (1.0 mg L^{-1}), BA (1.0 mg L^{-1}), GA_3 (1.0 mg L^{-1}); *L*, Put (2.0 mg L^{-1}), BA (2.0 mg L^{-1}), GA_3 (2.0 mg L^{-1}); *M*, Spd (1.0 mg L^{-1}), BA (1.0 mg L^{-1}), Kn (1.0 mg L^{-1}); *N*, Spd (2.0 mg L^{-1}), BA (2.0 mg L^{-1}), Kn (2.0 mg L^{-1}); *O*, Spd (1.0 mg L^{-1}), BA (1.0 mg L^{-1}), GA_3 (1.0 mg L^{-1}); *P*, Spd (2.0 mg L^{-1}), BA (2.0 mg L^{-1}), GA_3 (2.0 mg L^{-1}) and *Q*, control (MS0). Data was collected after 4-5 wk of shoot culture. Values are mean of three replicates with standard error (± S.E.), *bars with common letters* were significantly different at $P < 0.05$.

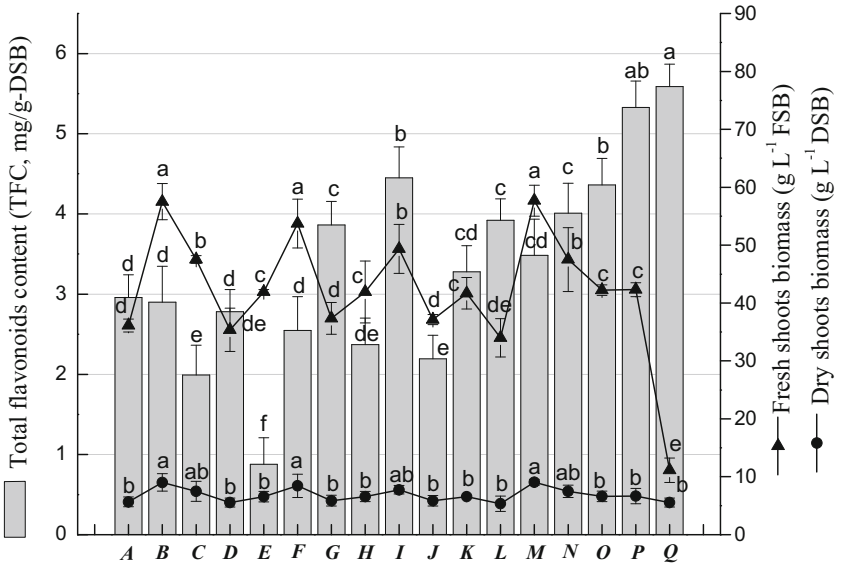

Figure 3. Total flavonoids content and its correlation with fresh and dry biomass in shoots treated with *A*, Put (1.0 mg L^{-1}), BA (1.0 mg L^{-1}); *B*, Put (2.0 mg L^{-1}), BA (2.0 mg L^{-1}); *C*, Put (1.0 mg L^{-1}), Kn (1.0 mg L^{-1}); *D*, Put (2.0 mg L^{-1}), Kn (2.0 mg L^{-1}); *E*, Spd (1.0 mg L^{-1}), BA (1.0 mg L^{-1}); *F*, Spd (2.0 mg L^{-1}), BA (2.0 mg L^{-1}); *G*, Spd (1.0 mg L^{-1}), Kn (1.0 mg L^{-1}); *H*, Spd (2.0 mg L^{-1}), Kn (2.0 mg L^{-1}); *I*, Put (1.0 mg L^{-1}), BA (1.0 mg L^{-1}), Kn (1.0 mg L^{-1}); *J*, Put (2.0 mg L^{-1}), BA (2.0 mg L^{-1}), Kn (2.0 mg L^{-1}); *K*, Put (1.0 mg L^{-1}), BA (1.0 mg L^{-1}), GA_3 (1.0 mg L^{-1}); *L*, Put (2.0 mg L^{-1}), BA (2.0 mg L^{-1}), GA_3 (2.0 mg L^{-1}); *M*, Spd (1.0 mg L^{-1}), BA (1.0 mg L^{-1}), Kn (1.0 mg L^{-1}); *N*, Spd (2.0 mg L^{-1}), BA (2.0 mg L^{-1}), Kn (2.0 mg L^{-1}); *O*, Spd (1.0 mg L^{-1}), BA (1.0 mg L^{-1}), GA_3 (1.0 mg L^{-1}); *P*, Spd (2.0 mg L^{-1}), BA (2.0 mg L^{-1}), GA_3 (2.0 mg L^{-1}) and *Q*, control (MS0). Data was collected after 4–5 wk of shoot culture. Values are mean of three replicates with standard error (± S.E.), *bars with common letters* were significantly different at $P < 0.05$.

compared to the control. Furthermore, the average number of roots per shoot (30.8) was observed on medium fortified with combinations of Put, IBA, and IAA (1.0 mg L^{-1}) as compared to the control (2.4). Therefore, it is concluded that different concentrations and combinations of auxin and PAs showed maximum effect on the multiplication of roots per plantlet

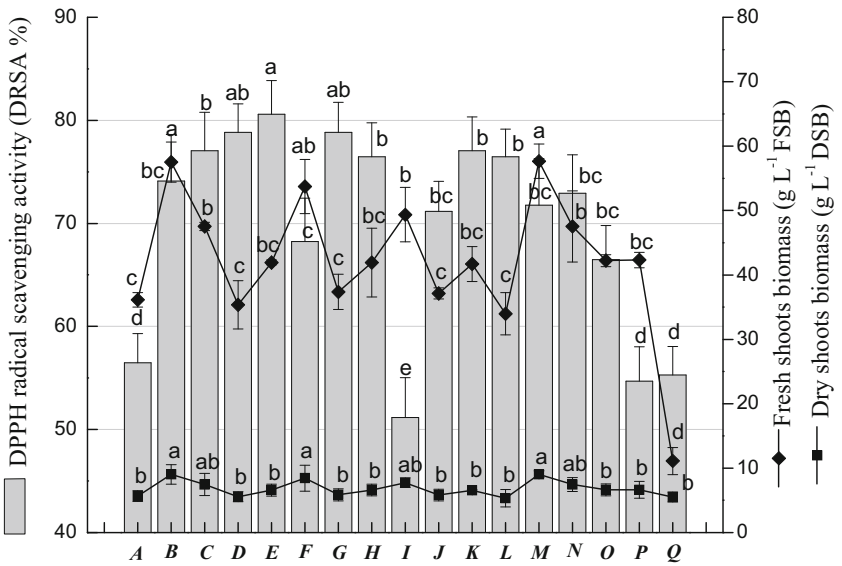

Figure 4. DPPH radical scavenging activity and its correlation with fresh and dry biomass in shoots treated with *A*, Put (1.0 mg L^{-1}), BA (1.0 mg L^{-1}); *B*, Put (2.0 mg L^{-1}), BA (2.0 mg L^{-1}); *C*, Put (1.0 mg L^{-1}), Kn (1.0 mg L^{-1}); *D*, Put (2.0 mg L^{-1}), Kn (2.0 mg L^{-1}); *E*, Spd (1.0 mg L^{-1}), BA (1.0 mg L^{-1}); *F*, Spd (2.0 mg L^{-1}), BA (2.0 mg L^{-1}); *G*, Spd (1.0 mg L^{-1}), Kn (1.0 mg L^{-1}); *H*, Spd (2.0 mg L^{-1}), Kn (2.0 mg L^{-1}); *I*, Put (1.0 mg L^{-1}), BA (1.0 mg L^{-1}), Kn (1.0 mg L^{-1}); *J*, Put (2.0 mg L^{-1}), BA (2.0 mg L^{-1}), Kn (2.0 mg L^{-1}); *K*, Put (1.0 mg L^{-1}), BA (1.0 mg L^{-1}), GA_3 (1.0 mg L^{-1}); *L*, Put (2.0 mg L^{-1}), BA (2.0 mg L^{-1}), GA_3 (2.0 mg L^{-1}); *M*, Spd (1.0 mg L^{-1}), BA (1.0 mg L^{-1}), Kn (1.0 mg L^{-1}); *N*, Spd (2.0 mg L^{-1}), BA (2.0 mg L^{-1}), Kn (2.0 mg L^{-1}); *O*, Spd (1.0 mg L^{-1}), BA (1.0 mg L^{-1}), GA_3 (1.0 mg L^{-1}); *P*, Spd (2.0 mg L^{-1}), BA (2.0 mg L^{-1}), GA_3 (2.0 mg L^{-1}) and *Q*, control (MS0). Data was collected after 4–5 wk of shoot culture. Values are mean of three replicates with standard error (± S.E.), *bars with common letters* were significantly different at $P < 0.05$.

(Table 3). It is reported that MS medium augmented with NAA (2.0 mg L^{-1}) produced the highest number of roots per plantlets (26.3) (Aman *et al.* 2013). Chotikadachanarong and Srisuluk (2013) observed that the addition of NAA to media produced 11.2 roots per shoot in Stevia. The current observation showed that maximum number of roots per shoot (30.8) and the higher rooting frequency were attributed to PAs in the media. In earlier experiments, only auxin was used for root multiplication in Stevia species. Regenerated plantlets were successfully transferred to pots containing combination of soil, sand, and manure in a ratio of 2:1:1 (Fig. 1i). The pots were kept in a growth chamber for 5 wk and then shifted to the greenhouse for hardening. Thereafter, healthy plantlets from pots were successfully transferred to field conditions for survival as T_1 generation. These plants will be used for T_2 generation because of their vigorous growth, higher number of leaves, and improved steviol glycosides.

Quantification of stevioside contents by HPLC In the present investigation, highest stevioside content (10.2 mg/g DSB) was recorded in shoots regenerated on MS media augmented with synergistic combination of Spd (2.0 mg L^{-1}), BA (2.0 mg L^{-1}), and Kn (2.0 mg L^{-1}) as compared to control (3.02 mg/g DSB, Table 4). Moreover, shoots collected from medium containing combinations of Spd (1.0 mg L^{-1}) and Kn (1.0 mg L^{-1}) produced the highest stevioside content (6.07 mg/g DSB). It showed that synergistic combination of Spd, BA, and Kn enhanced stevioside content as compared to the previous reports (Table 5). Aman *et al.* (2013) recently observed 0.08-mg/g content of stevioside in shoots *in vitro* on medium containing combination of BA (3.0 mg L^{-1}) and Kn (3.0 mg L^{-1}). In this study, we obtained a maximum stevioside content of 10.2-mg/g DSB, as compared to control and previous reports. Dey *et al.* (2013) observed that the addition of chlorocholine chloride and IBA enhanced stevioside content (2.9 mg/g FW) but they were also lower than in this report. Furthermore, Reis *et al.* (2011) did not reported stevioside content in adventitious root culture of *S. rebaudiana*. In contrast, Ladygin *et al.* (2008) reported trace stevioside contents in calli (0.06 mg/g DW) and shoots (0.3 mg/g DW) of *S. rebaudiana*. Mathur and Shekhawat (2012) reported a lower stevioside content (0.4 mg/g DW) in cell suspension culture of *S. rebaudiana* by using BA and NAA. However, Woelwer-Rieck *et al.* (2010) observed a higher stevioside content (8.7 mg/g DW) in the leaves of intact plants. It showed that the level of PAs and cytokinin-stimulated steviosides were least in *in vitro* cells and higher in tissues and leaves of intact plant. Moreover, biomass-independent stevioside production was observed in different treatments. Maximum FSB (57.5 and 57.7 g L^{-1}) and DSB (9.03 and 9.05 g L^{-1}) were observed using the combination of BA + Put (2.0 mg L^{-1}) and BA + Kn + Spd (1.0 mg L^{-1}), but these combinations showed less effective response in stevioside enhancement (Table 4). It is concluded that stevioside production was PGR dependent and biomass independent. In agreement with our data, Ali and Abbasi (2014) also reported PGRs dependent and biomass independent active compounds production in *Artemisia absinthium*.

Content of total phenolics and flavonoids In the present investigation, higher TPC (1.8 mg/g DSB) was observed with *in vitro* shoots treated with synergistic combinations of Spd, BA, and GA_3 (2.0 mg L^{-1}) as compared to the control (0.9 mg/g DSB). The combination of Spd and Kn (1.0 mg L^{-1}) also influenced TFC (1.3 mg/g DSB) more than control (Fig. 2). It has been concluded from these results that TPC production is also PGR dependent rather than biomass accumulation. Furthermore, TFC did not show linear correlation with biomass accumulation, PAs, and PGRs application, rather higher TFC (5.6 mg/g DSB) was observed on PGR-free medium (Fig. 3). These results are in agreement with the reports of Ali and Abbasi (2014) and Ahmad *et al.* (2014) who observed PGR-dependent production of phenolic content in different tissues of *Artemisia absinthium* and *Piper nigrum*, respectively.

Free radical scavenging activity The DPPH radical scavenging activity (DRSA) was investigated in shoots *in vitro* after the application of PAs and PGRs. The shoots grown by the combination of Spd and BA (1.0 mg L^{-1}) displayed maximum value of DRSA (80.6%) as compared to control (55.3%) and other treatments (Fig. 4). A positive correlation of DRSA with the application of PAs and PGRs was observed. The current results are in line with the reports of Abbasi *et al.* (2010), Ahmad *et al.* (2010; 2014) and Ali and Abbasi (2014).

In conclusion, the addition of various combinations of PAs and auxin/cytokinin promoted morphogenetic potential. Furthermore, the current results showed that stevioside content, phenolics, flavonoids, and antioxidant activity in shoots *in vitro* was positively regulated by synergistic combinations of polyamines and cytokinin rather than biomass accumulation. These results have the potential to be exploited for pharmaceutical and other health industries on commercial bases.

Acknowledgment The authors are highly thankful to the Pakistan Science Foundation (PSF) for the financial support to complete this research work.

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