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Enhanced micropropagation in *Stevia rebaudiana* Bertoni

BILQUEES FATIMA[1], ZAHID NAZIR[1], MUHAMMAD USMAN*[1], SYEDA ANUM MASOOD BOKHARI[1] & SHUMAILA[2]

[1]Plant Tissue Culture Cell, Institute of Horticultural Sciences, University of Agriculture, Faisalabad-38040, Pakistan
[2]Department of Botany, University of Agriculture, Faisalabad-38040, Pakistan

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***Corresponding Author:**

Muhammad Usman:
m.usman@uaf.edu.pk

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ABSTRACT

Stevia is a natural sweet herb grown as a substitute for sugar producing crop. Its leaves have stevioside and have about 300 times more sweetness compared with other sugar producing crops. Its seeds are small and poor in germination hence can't be used for mass propagation. Hence, this study was aimed to enhance propagation efficiency using Murashige and Skoog (MS) basal media supplemented with benzyl aminopurine (BAP), kinetin, indole acetic acid (IAA) and naphthalene acetic acid (NAA) in different combinations. Days to shoot induction (SI) were reduced to 4.6-5.6 days on M_3 media (BAP + NAA) compared with M_4 media (BAP and IAA) and mean shoot induction was greater on M_3 media. Number of shoots induced per explant was higher (6 shoots/explant) on M_1 medium containing higher levels of BAP and M_3 media containing BAP and NAA (2.50 + 0.50 mgL^{-1}). Shoot elongation, internodal distance, number of leaves, plant fresh weight, plant dry weight, and leaf fresh weight were higher at higher levels (2.50 + 0.50 mgL^{-1}) of medium containing BAP + NAA (M_3) and BAP + IAA (M_4), respectively. Maximum root induction (83.33 %), a higher number of roots and more root length were found on M_3 medium containing BAP and NAA (2.50 + 0.50 mgL^{-1}). It is concluded that M3 medium containing BAP and NAA was better for enhanced shoot multiplication in stevia. The multiplied plant material was transferred to green house after acclimatization for further plant growth and biochemical studies. Such studies shall be useful for rapid clonal multiplication of the desired genotypes in stevia.

Keywords: Auxins, Clonal propagation, Cytokinin, Shoot induction, Organogenesis, Sweet herb

INTRODUCTION

Stevia (*Stevia rebaudiana* Bertoni) is a natural perennial sweet herb, a member of family Asteraceae and grown as a substitute for sugar producing crops (Khiraoui et al., 2017). Its leaves are sweet having stevioside, a glycoside, and may contain it about 300 times more compared with other sugar producing crops (Ahmed et al., 2007; Chatsudthipong & Muanprasat, 2009; Lemus-Mondaca *et al.*, 2012). Stevia comprises 230 species, however, stevioside is produced only in two species viz. *phlebophylla* and *rebaudiana* (Guleria & Yadav, 2013). Stevioside is a low-caloric sweetener, hence, it is medicinally important for diabetic patients and overweight people. Its leaves have antimicrobial properties (Belda-Galbis et al., 2014), antioxidants (Criado et al., 2014), could cure many other diseases, and strengthen the immune system (Goyal et al., 2010). Stevia is indigenous to areas having cold climate including South America and grows wild in plateaus of Amambay near the borderline of Brazil and Paraguay. It is commercially grown in China, Japan, Taiwan, Thailand, Korea, India and Malaysia (Jain et al., 2009). Stevioside is mainly used as a natural sweetener in the food and pharmaceutical industry of Japan.

Seeds of stevia are small, have low storage life, poor germination (Yadav et al., 2011; Lemus-Mondaca, 2012) and progeny may have variable stevioside contents (Bespalhok-Filho & Hattori, 1993; Rathi and Arya, 2009) hence, seeds could not be used for mass propagation. Few genotypes also show self-incompatibility leading to poor seed setting (Raina et al., 2013). Its cultivation is habitat specific; plants need proper soil and climatic conditions for asexual propagation by cuttings (Mishra et al., 2010; Singh et al., 2012). *In vitro* clonal propagation offers efficient mass scale plant production in woody and

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herbal crops having propagation issues using nodal or internodal explants (Usman et al., 2005; Thiyagarajan & Venkatachalam, 2012; Pereira et al., 2017). *In vitro* propagation of stevia has been reported by researchers in different varieties (Ali et al., 2010; Anbazhagan et al., 2010). Large number of stevia plants of good quality could be produced within an extended period (Singh et al., 2017). Nodal segments have been used for *in vitro* clonal propagation (Aziz & Khaled, 2017; Singh et al., 2017; Thilakarathne et al., 2019) and media supplementation with different phytohormones have further hastened development of good horticultural traits and stevioside levels in leaves of *in vitro* raised plants (Jain et al., 2014). An efficient micropropagation protocol is required to regenerate homozygous plants of superior quality in desired genotypes of stevia as micropropagation could be genotype dependent (Das et al., 2011; Razak et al., 2014). Hence, this study was aimed to micropropagate stevia and enhance propagation efficiency using different plant growth regulators (PGRs).

MATERIALS AND METHODS

Plant material, inoculation and culture conditions

Shoot cuttings were collected from six months old stevia plants taken from Ayub Agriculture Research Institute (AARI), Faisalabad and growing under greenhouse conditions in the Institute of Horticultural Science, UAF. Nodal segments (1 cm long) were prepared and washed under running tap water to remove dust particles. The explants were surface sterilized in 70% ethanol for 3 minutes and rinsed 3-5 times with sterile water. Explants were dipped in 5% sodium hypochlorite solution plus 1-2 drops of Twen-20 for 5 minutes and rinsed 3-5 times with sterile water. Murashige and Skoog 'MS' (1962) medium supplemented with different levels of cytokinins including benzyl amino purine (BAP), Kinetin (Kn) and auxins [indole acetic acid (IAA) and naphthalene acetic acid (NAA)] alone and in combinations were used to induce multiple shoot induction and subsequent rooting (Table 1). Sucrose (30 gL^{-1}) was used as a carbon source and 8 gL^{-1} agar (Phytotech, USA) was used as a solidifying agent in the media. Medium pH was adjusted to 5.7-5.8. Media was sterilized following standard autoclave conditions. The explants were cultured under sterile conditions in the inoculation chamber and placed in growth room maintained at 25±2 °C temperature and 2500 lux light intensity with 16 hrs. Photoperiod for 6-8 weeks (Butt et al., 2013)

Experimental layout and data analysis

Each experiment consisted of 20 tubes per treatment and having one explant per tube. Data were collected for parameters as days to shoot induction, shoot induction (%), number of shoots per explant, shoot length, internodal distance, number of leaves, days to root induction, root induction (%), root length, plant fresh weight, plant dry weight and leaf fresh weight. The experiment was laid out in completely randomized design (CRD). Analysis of variance (ANOVA) was employed to test significance of the data using MSTATC software. Least significance difference (LSD) test was used to compare treatment means (Steel et al., 1997).

Table I: Murashige and Skoog (MS) basal media formulations for micropropagation

Treatments	MS basal media + plant growth regulators (PGRs) (mgL^{-1})				
	MS0 (M_0)	BAP (M_1)	Kn (M_2)	BAP + NAA (M_3)	BAP + IAA (M_4)
T_o	MS media	-	-	-	-
T_1	-	0.50	0.50	1.00 + 0.50	1.00 + 0.50
T_2	-	1.00	1.00	1.50 + 0.50	1.50 + 0.50
T_3	-	1.50	1.50	2.00 + 0.50	2.00 + 0.50
T_4	-	2.00	2.00	2.50 + 0.50	2.50 + 0.50
T_5	-	2.50	2.50	-	-

RESULTS

Shoot induction (SI) and growth responses

Days to SI were highly reduced to 4.6-5.6 on M_3 media containing different levels of BAP and NAA compared with other PGRs and MS0 (devoid PGRs) media (8.3 days). Shoot induction (%) increased with rising levels of PGRs in all media M_1-M_4 (66% - 94%) compared with MS0 media (55%), however, overall SI was higher in M_3 media (Table 2). Number of shoots induced per explant was markedly higher (6 shoots/explant) at higher levels of BAP alone (2.50 mgL^{-1}) and BAP in combination with NAA (2.50 +

0.50 mgL^{-1}) compared with MS0 media (1.55 shoots/explant) as shown in Fig. 1c-f. Shoot elongation, internodal distance, number of leaves, plant fresh weight (PFW), plant dry weight (PDW) and leaf fresh weight (LFW) were higher on M_3 and M_4 media having auxins in combination with BAP compared with M_1 and M_2 media lacking auxins. Amongst M_3 and M_4 media containing BAP and auxins (NAA and IAA), M_3 media (BAP + NAA) was found better for inducing a greater number of shoots/explant, other shoot growth and plant weight parameters discussed above.

Root induction (RI) and growth responses

Rooting was induced only on M_3 and M_4 media having BAP in combination with auxins (NAA and IAA) while there was no rooting in shoots induced on M_1 and M_2 media having BAP and Kn alone. The number of roots/explant (Fig. 2a) and root length (Fig. 3a) were higher on M_3 media compared with M_4. Percent root induction was earlier and initiated after 20-24 days of culture on M_3 media compared with M_4 media (24-26 days) as shown in Fig. 2b. Root induction (%) was higher (75%-83%) on M_4 compared with M_3 media (58%-83%) as shown in Fig. 3b.

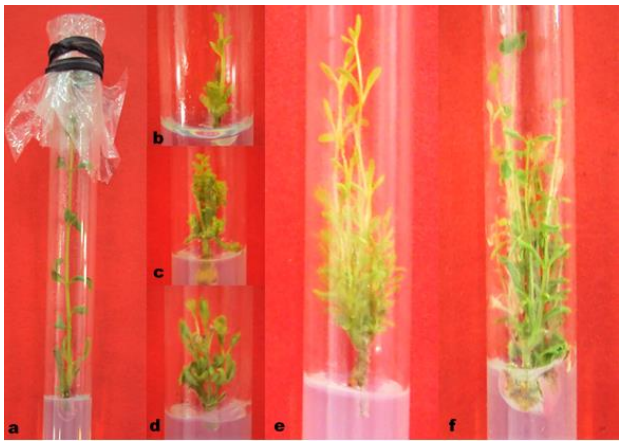

Fig 1: Multiple shoot induction responses in *Stevia rebaudiana* Bertoni on Murashige and Skoog (MS) media modified with different PGRs. Figures show a) shoot growth on MS0 media (control), b) shoot induction on BAP 1 mgL^{-1}, c, e) shoot induction and growth on BAP 2.5 mgL^{-1} and d, f) BAP + NAA 2.5 + 0.5 mgL^{-1}.

DISCUSSION

Cytokinin like BAP, Kn and Zeatin are known for inducing shoots while auxins including NAA and IAA are root inducers, *in vitro* (Su et al., 2011). Com binations of cytokinin and auxin may lead to variable organogenic responses. In the current study, shoot induction responses were observed when cytokinin including BAP and Kn were used in the media. A combination of the cytokinin showing better response-BAP was used in combination with auxins (NAA and IAA) which induced both shoots and subsequent rooting. Axillary nodal segments have been used for direct shoot induction and growth in stevia (Das et al., 2011). Better shoot induction and growth was observed in media containing BAP, Kn, and half-strength MS media and the addition of NAA with BAP showed poor shoot proliferation.

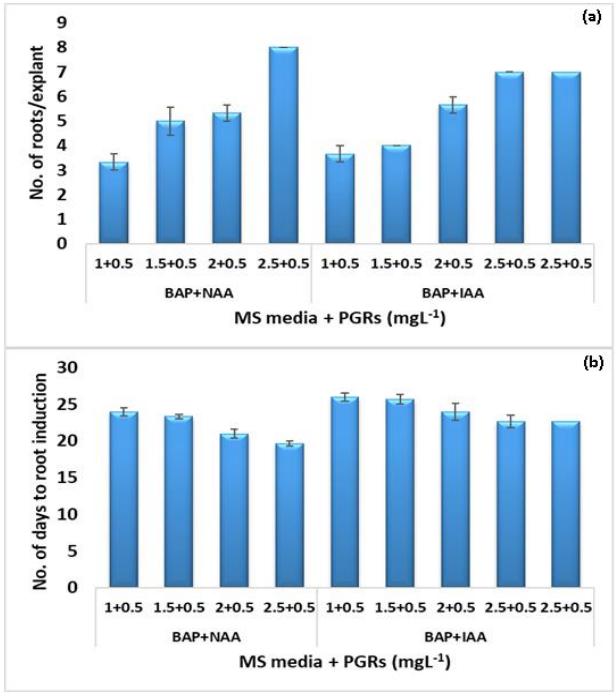

Fig 2: Effect of MS media supplemented with different levels of PGRs on number of roots per explant (a) and days to root induction (b) in *Stevia rebaudiana* Bertoni.

Table II: Effect of plant growth regulators on shoot induction (SI) and growth in *Stevia rebaudiana* Bertoni

MS basal media + plant growth regulators (mgL^{-1})		Days to shoot induction	Shoot induction (%)	Number of shoots/explant	Shoot length (cm)	Internodal distance (cm)	Number of leaves/shoot	PFW* (g)	PDW* (g)	LFW* (g)
MS0 (control)		8.33 ± 0.57^{d}	55.00 ± 4.43^{d}	1.55 ± 0.57^{d}	1.81 ± 0.04^{l}	0.70 ± 0.00^{i}	2.66 ± 0.33^{e}	0.064 ± 0.001^{i}	0.034 ± 0.001^{fg}	0.024 ± 0.001^{g}
BAP (M_1)	0.50	7.00 ± 0.57^{bcd}	80.56 ± 10.00^{ab}	3.00 ± 0.57^{bcd}	2.17 ± 0.04^{ijk}	0.76 ± 0.00^{fghi}	0.76 ± 0.00^{fghi}	0.065 ± 0.001^{i}	0.033 ± 0.001^{g}	0.027 ± 0.001^{efg}
	1.00	6.33 ± 0.66^{abcd}	77.76 ± 2.76^{a}	3.66 ± 0.66^{bcd}	2.30 ± 0.05^{hij}	0.81 ± 0.01^{efg}	0.81 ± 0.01^{efg}	0.068 ± 0.001^{ghi}	0.040 ± 0.001^{cd}	0.029 ± 0.001^{ef}
	1.50	5.66 ± 0.33^{bcd}	83.33 ± 8.33^{ab}	3.33 ± 0.33^{bcd}	2.37 ± 0.01^{hi}	0.87 ± 0.01^{cde}	0.87 ± 0.01^{cde}	0.073 ± 0.001^{d-g}	0.040 ± 0.001^{cd}	0.033 ± 0.001^{cd}
	2.00	5.66 ± 0.33^{abc}	88.88 ± 5.55^{a}	4.66 ± 0.33^{bc}	2.47 ± 0.01^{gh}	0.96 ± 0.01^{bc}	0.95 ± 0.01^{bc}	0.075 ± 0.001^{c-f}	0.042 ± 0.001^{bc}	0.034 ± 0.001^{bcd}
	2.50	5.33 ± 0.33^{ab}	94.44 ± 5.55^{a}	6.66 ± 0.33^{a}	2.63 ± 0.01^{fg}	1.05 ± 0.02^{a}	1.05 ± 0.02^{a}	0.079 ± 0.001^{cd}	0.043 ± 0.001^{bc}	0.038 ± 0.001^{ab}
Kinetin (M_2)	0.50	7.33 ± 0.57^{d}	75.00 ± 4.43^{cd}	2.00 ± 0.57^{d}	1.87 ± 0.01^{l}	0.70 ± 0.00^{i}	0.70 ± 0.00^{i}	0.064 ± 0.001^{i}	0.034 ± 0.001^{g}	0.026 ± 0.001^{fg}
	1.00	7.00 ± 0.57^{bcd}	83.33 ± 8.33^{a}	2.66 ± 0.33^{cd}	2.02 ± 0.01^{kl}	0.72 ± 0.01^{hi}	0.72 ± 0.01^{hi}	0.066 ± 0.001^{hi}	0.035 ± 0.001^{efg}	0.025 ± 0.001^{fg}
	1.50	6.66 ± 0.33^{cd}	83.33 ± 8.33^{a}	2.66 ± 0.33^{cd}	2.12 ± 0.01^{jk}	0.75 ± 0.01^{ghi}	0.74 ± 0.01^{ghi}	0.068 ± 0.001^{ghi}	0.037 ± 0.001^{d-g}	0.029 ± 0.001^{ef}
	2.00	6.50 ± 0.33^{abcd}	91.66 ± 8.33^{a}	3.66 ± 0.33^{bcd}	2.24 ± 0.01^{ijk}	0.81 ± 0.01^{efgh}	0.80 ± 0.01^{efgh}	0.069 ± 0.001^{f-i}	0.038 ± 0.001^{c-f}	0.031 ± 0.001^{de}
	2.50	6.00 ± 0.28^{cd}	75.00 ± 4.43^{cd}	2.50 ± 0.28^{cd}	2.33 ± 0.01^{hijk}	0.85 ± 0.01^{ef}	0.85 ± 0.01^{ef}	0.075 ± 0.001^{cde}	0.040 ± 0.001^{cd}	0.034 ± 0.001^{cd}
BAP + NAA (M_3)	1.00 + 0.50	5.66 ± 0.33^{bcd}	83.33 ± 8.33^{a}	3.33 ± 0.33^{bcd}	3.28 ± 0.04^{c}	0.96 ± 0.00^{b}	0.96 ± 0.00^{b}	0.072± 0.001^{e-h}	0.039± 0.001^{cde}	0.035 ± 0.001^{bc}
	1.50 + 0.50	5.33 ± 0.57^{abcd}	86.11 ± 7.35^{a}	4.00 ± 0.57^{bcd}	3.34 ± 0.03^{bc}	0.98 ± 0.00^{ab}	0.97 ± 0.00^{ab}	0.077 ± 0.002^{cde}	0.043 ± 0.001^{bc}	0.037 ± 0.001^{abc}
	2.00 + 0.50	5.00 ± 0.33^{abc}	94.44 ± 5.55^{a}	4.33 ± 0.33^{bcd}	3.56 ± 0.04^{b}	0.98 ± 0.00^{ab}	0.97 ± 0.00^{ab}	0.086 ± 0.002^{b}	0.047 ± 0.001^{a}	0.039 ± 0.001^{a}
	2.50 + 0.50	4.66 ± 0.57^{a}	94.44 ± 5.55^{a}	6.00 ± 0.57^{ab}	4.26 ± 0.14^{a}	0.98 ± 0.00^{ab}	0.98 ± 0.00^{ab}	0.099 ± 0.003^{a}	0.050 ± 0.001^{a}	0.041 ± 0.001^{a}
BAP + IAA (M_4)	1.00 + 0.50	6.66 ± 0.33^{cd}	83.33 ± 8.33^{a}	2.66 ± 0.33^{cd}	2.82 ± 0.01^{ef}	0.86 ± 0.02^{de}	0.86 ± 0.02^{de}	0.077 ± 0.001^{ghi}	0.037 ± 0.001^{d-g}	0.026 ± 0.001^{fg}
	1.50 + 0.50	5.66 ± 0.33^{cd}	66.66 ± 6.66^{d}	2.66 ± 0.33^{cd}	2.95 ± 0.02^{de}	0.94 ± 0.00^{bcd}	0.94 ± 0.00^{bcd}	0.067 ± 0.001^{ghi}	0.039 ± 0.001^{c-f}	0.028 ± 0.001^{efg}
	2.00 + 0.50	5.33 ± 0.33^{abc}	94.44 ± 5.55^{a}	4.33 ± 0.33^{bc}	3.17 ± 0.01^{cd}	0.99 ± 0.00^{ab}	0.99 ± 0.00^{ab}	0.075 ± 0.001^{c-f}	0.042 ± 0.001^{bc}	0.029 ± 0.001^{ef}
	2.50 + 0.50	5.00 ± 0.33^{abc}	94.44 ± 5.55^{a}	4.33 ± 0.33^{bc}	3.53 ± 0.03^{b}	1.01 ± 0.04^{ab}	1.01 ± 0.04^{ab}	0.081 ± 0.001^{bc}	0.046 ± 0.001^{ab}	0.031 ± 0.001^{de}

In current study, both shoot induction and rooting responses were better in M_3 media containing BAP and NAA (2.50 + 0.50 mgL^{-1}) compared with other media compositions. Kinetin was reported to induce better shoot induction in nodal stem sections and shoot apex while BAP was found better for shoot induction and proliferation in all the three explants types used (Chalapathi et al., 1997; Anbazhagan et al., 2010; Javed et al., 2019). BAP was also reported to induce less shoot growth and proliferation compared with Kn in a Malaysian study on Stevia. Shoot formation was higher on media containing BAP and Kn (Abdul-Razzak et al., 2014). In contrast, the number of shoots per explant was higher on media containing BAP (M_1), BAP and NAA (M_3) while shoot growth, internodal distance and plant fresh weight were higher on M_3 media. Our findings are in line with Shekhawat et al. (2015) who reported higher shoot induction on MS media containing BAP in Passiflora. The number of leaves was higher in plants growing on control media, however, leaf fresh weight was also higher in both M_1 and M_3 media indicating higher leaf size and growth on media containing NAA along with BAP. These findings are in line with the previous report of Alhady (2011)

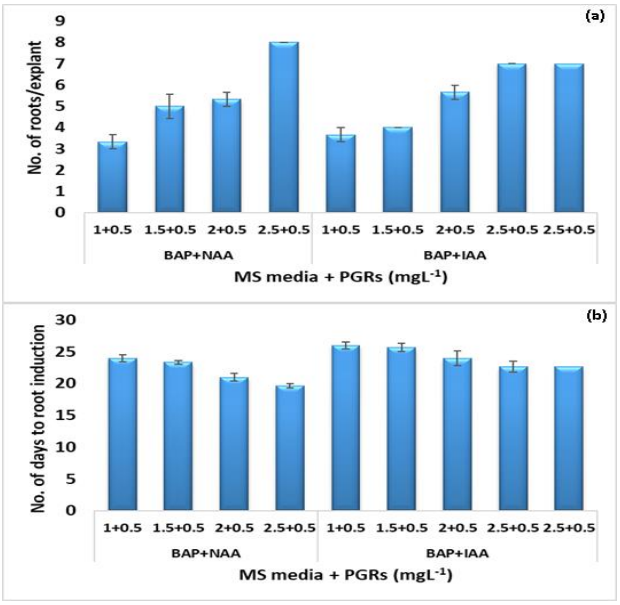

Fig 3: Effect of MS media supplemented with different levels of PGRs on root length (cm) (a) and root induction % (b) in *Stevia rebaudiana* Bertoni.

In micropropagation studies, root induction is usually more critical particularly in woody plants (Usman et al., 2005; Pereira et al., 2017) and some herbaceous plants like stevia. Usually auxins including NAA, IAA and IBA are used for root induction in difficult to root woody crops and stevia (Usman et al., 2005; Tolera et al., 2016; Tufail et al., 2019). Higher root length was reported in a medicinal shrub, *Holarrhena antidysenterica,* on media containing 0.25 mgL^{-1} NAA and its higher concentration enhanced root induction upto 90% (Kanungo et al., 2012). More effectiveness of NAA compared with IAA for rooting was also reported by Tolera et al. (2016). These findings are in line with our results as the number of roots was comparable in both M_3 and M_4 media, however, the use of NAA 0.5 mgL^{-1} as auxin source in M_3 media enhanced more root growth compared with media containing IAA (M_4). Plant material was acclimatized and transferred to green house for further plant growth. Conclusively, the optimized micropropagation protocol demonstrates greater potential of mass scale plant multiplication of stevia using BAP for a higher frequency of shoot induction and plant growth, whereas NAA for enhanced rooting.

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