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RESEARCH ARTICLE

In Vitro Morphogenesis of Stevia rebaudiana, A naturally occurring sweet plant

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Abstract

Stevia rebaudiana is likely to become as a major source of high potency sweetener for the growing natural food market worldwide. The present investigation was conducted to develop a fast and efficient protocol to propagate S. rebaudiana by tissue culture. Murashige and Skoog (MS) basal supplemented with 6-Benzylamino purine (BAP) at 13.5 µM and α-Napthaleneacetic acid (NAA) at 8.0 µM showed best performance as compared to (BAP) alone at 13.3 µM for bud breaking. While two different combinations of BAP at 13.3 µM with Adenine sulphate (ADS) at 61.8 µM and BAP at 13.5 µM with (NAA) at 8.0 µM in MS basal medium resulted in maximum shoot regeneration and development. The combination of (NAA) at 10.7 µM with (BAP) at 8.8 µM resulted in the highest percentage (100%) of callus initiation. The best rooting was observed with (NAA) at 16.1 µM with (BAP) at 10.7 µM than Indole Acetic Acid (IAA) at 16.1 µM alone.

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Introduction

Stevia rebaudiana, is a perennial herb belongs to the Asteraceae family. It is a natural sweetener plant known as "Sweet Weed", "Sweet Leaf", "Sweet Herbs" and "Honey Leaf", which is estimated to be 300 times sweeter than cane sugar (Chalapathi and Thimmegowda, 1997), (Liu. and Li, 1995). Stevia grows to about 50-65 cm tall, with sessile, oppositely arranged lanceolate to oblanceolate leaves, serrated towards apex. It is one of 154 members of the genus Stevia, which produces sweet steviol glycosides like stevioside, rebaudioside A, rebaudioside C and dulcoside A. Pure extract of stevioside is non-caloric and 300 times sweeter than suger (Bhosle, 2004) unlike many low-caloric sweetners, stevioside is stable at high temperatures and over a range of pH values from 3 to 9 (Kinghorn and Soejarto, 1985). Stevia is a major source of high potency sweetener for hundreds of years. The useful part of stevia rebaudiana is the leaves (Soejarto et al, 1982). Stevia rebaudiana (Bertoni), contains in its leaves all of the eight ent-kaurene glycosides that produce the sweet taste sensation with stevioside being the major constituent (Kinghorn et al., 1984). The sweetness in leaf is due to the presence of an intensive-sweetening agent called stevioside. Stevioside is regenerated as a valuable natural sweetening agent because of its relatively good taste and chemical stability (Yamazaki and Flores, 1991), (Toyoda and Matsui, 1997).

Successful exploitation of medicinal plants can be used as a key to address the vast number of problems associated with varied health, nutritional and social aspects of human being. Stevia rubidiana has a special importance as it is also non-caloric, non-fermentable and does not darken upon cooking (Crammer and Ikan, 1986). . It is suited for both diabetic patients, as well as for obese persons intending to lose weight by avoiding sugar supplement in the

diet. No allergic reactions seem to exist (Geuns, 2003). Stevia has no calcium cyclamate, no saccharin, no aspartame, and no calories therefore it is safe for diabetics, as it does, not affect blood sugar levels; it does not have the neurological or renal side effects of some of the artificial sweeteners. Stevia being used as a sweetener is likely to become a major source of high potency sweetener for the growing natural food market worldwide (Matsui et al, 1996) (Shen and Xu, 1997). Seeds of stevia show very less vigour and do not allow the production of homogenous population which leads to variability in sweetening level and composition (Felippe et al., 1971; Miyagawa et al., 1986).

The conventional methods of cultivation or propagation of Stevia is time consuming, unpredictable, unreliable and less productive. Therefore, there is a crucial need to develop method for rapid multiplication of Stevia. Seed germination of Stevia is often poor and less productive. Poor seed germination percentage is the limiting factor to large scale cultivation of this species (Tamura et al., 1984). Due to instability of plantlet produced through stem cutting, micropropagation method may overcome many of the limitations associated with conventional method and can be used for rapid multiplication. Thus, the present study was undertaken to develop and establish a reproducible protocol for plantlet regeneration and to determine the suitable concentration for in vitro seedling production.

Materials and Methods

An experiment on in vitro callus induction and subsequent plantlet regeneration of Stevia was conducted at the Biotechnology laboratory, College of Biotechnology, Birsa Agricultural University Ranchi, Jharkhand. Healthy plants of 2-7 week growing in the mother nursery were used for the present study (fig. 1). Modified Murashige and Skoog media was used for culture of the explants (Murashige and Skoog, 1962). Proper sterilization of MS medium, test tubes, pipette, petridishes, beakers, scalpels, forceps, needles and other glasswares and growth chamber were made. Incubation chamber was properly maintained. Exogenous hormone, like, 6-Benzyleaminopurine (BAP), Adenine sulphate (ADS) Napthyl Acetic Acid (NAA) and Indole Acetic Acid (IAA) were used in different concentration and combinations. (ADS) was used as an additive.

The nodal and intermodal explants were cut into small pieces (about 0.5-0.6 cm long) and then treated with 2% savlon with constant shaking for 5-6 minutes and washed thoroughly with distilled water. Then the explants were taken under Laminer Air Flow Cabinet. The surface sterilization of the explants was done with 0.2% mercuric chloride solution for 8 minutes under aseptic condition followed by washing 5-6 times with sterilized distilled water. The explants were inoculated on MS medium for Bud breaking. The experiment was set up in five replicates and repeated thrice.

The cultures were grown under 3000 lux light from Phillips fluorescent day tube for 16/8 photoperiod. The ambient temperature was maintained at 25 ± 2 ^{o}C and the relative humidity was adjusted to approximately 55-65%.

The shoot tips were sub cultured at an interval of 15 days to overcome excessive phenolic exudation. The induced cultures were sub cultured at regular interval of every 20 days. The plantlets having fully developed 2-4 leaves were then transferred to the rooting medium for the initiation and development of roots. The growth index rate was measured at 15 days interval, starting from 15^{th} day up to 60^{th} day. To take initial and final weight, each bottle containing media were weighted i.e., before and after the inoculation. This difference was worked out in order to get the weight of calli. One bottle containing same amount of medium only, was kept as control to assess the loss of weight of medium due to drying up. Effects of different treatments were observed in responses to the culture. Data recorded represented the mean percentage and mean number.

The effect of different concentration treatment of cytokine (BAP) on bud proliferation after four weeks of inoculation were noted and expressed in percentage. Shoot multiplication was optimized by incorporating MS basal medium with different concentration and combination of BAP in combination with ADS. In vitro root initiation was studied under the influence of varying concentrations of NAA and BAP incorporation in MS basal medium. The observations were subsequently recorded as shootlets (%) showing rooting and number of roots per shootlets.

Callus produced by different explants viz. incorporation in the growth medium were studied and recorded as percentage value in the growth index. The regenerated plantlets were transferred to green house condition in pots filled with coco peat and sand for the purpose of primary hardening. Plants were put in green house conditions for at least (15-20) days. After the establishing of the roots the plants were transferred in the poly bags filled with soil for the purpose of secondary hardening.

Results and Discussion

The survival percentage of explants sub-cultured with respect to and without sub-culture as shown in (Table 1). It was observed that younger explants (one week to three week old) exude comparatively more phenolic compounds than the older explants (four week to seven week old). It ultimately affected the survival percentage of inoculated explants in media containing different hormonal combinations. The higher exudating explants showed lower percentage of survival than the lower exudating explants. The survival percentage was found to be best in the four-week old explants after the sub culturing.

Bud Breaking & Shoot Formation.

1) Bud breaking and shoot formation was observed in different phytohormonal combinations, such as BAP, BAP + NAA and BAP + ADS (Table 2). The maximum percentage of bud breaking and shoot formation per explant was observed in the medium containing BAP (13.3 µM) + ADS (61.82 µM) and BAP (13.3 µM) + NAA (8.0 µM) respectively (Table 2; Fig. 2a & 2b).

The effect of Bud breaking (%) and Shoot multiplication was also studied by varying the concentration of BAP with respect to time as shown in (Table 3). The concentration of BAP was varied between (1.11 to 13.3 µM). The highest Bud breaking (%) and shoot multiplication was recorded after six weeks of culturing with BAP (13.3 µM) and NAA of (10.7 µM) on the explant of Stevia rebaudiana in MS medium (Fig. 3). The exogeneous hormone 6-Benzyladenine (8.87 µM) and Indole-3-acetic acid (5.71 µM) have been successfully employed by (Sivaram and Mukundan, 2003) to promote of shoot apex.

Callus Response

The phytohormones NAA and BAP were used alone and in combinations (Table 4). Callus initiation occurred after (21 to 30) days of inoculation of leaves. The best callusing was observed after four weeks in BAP (13.3 µM) + NAA (10.7 µM) as shown in (Fig. 4a and 4b).

The role of phytohormones could be more prominent for callus induction if a well suited carbon source is selected. Studies show that basic culture medium and carbon sources effect the callus formation and morphogenesis considerably. Therefore, the combination of phytohormones along with the carbon source would be promising venue to be explored in the future studies. The role of other phytohormones such as 2, 4-D needs to be explored in order to get an efficient regeneration of callus. (Carlos and Kazumi, 1997) observed that maximum embryogenic callus formation occurred in medium supplement with 9.05 mM 2-4-D without kinetin. (18.10 mM) 2,4-D supplemented medium the best treatment was 2.32 mM kinetin. Further study in this direction is needed to increase the efficiency of the callus formation.

Root Formation

Root formation was observed in NAA, BAP combinations of phytohormones (Table 5). The root formation started after three and four weeks of inoculation of shootlets in rooting media which contained higher percentage of auxin as compared to cytokinin. The maximum percentage of root formation was observed in NAA (16.1 µM) + BAP (10.7 µM) at six week after inoculation in rooting media (Table 5, Fig. 5). The number of roots observed per explant varied from (3.0-10.0) at four weeks, and (5.0-15.0) at six weeks (Table 5). Similar observations were also reported by (Sivaram and Mukundan, 2003) that rooting of the in vitro-derived shoots could be achieved following subculture onto auxin-containing medium.

Survival Percentage

After the emergence of roots the plants were transferred to mist chamber and kept there for two weeks in pots filled with coca peat and sand. They were subsequently transferred to net house for hardening. The survival percentage was observed to be (70.0%) represented in (Fig. 6). These finding are in conformity with the result of (Sivaram and Mukundan, 2003), who showed that up to 70.0% of the plants survived from the hardening phase on the coco peat substrate.

Table 1 Effect of age, phenolic exudation and sub-culturing on survival percentage of explants.

Age of explants weeks	Degree of phenolic exudation	Survival % (without sub culturing)	Survival % (with sub culturing at each 15 days interval)
01	++++	23.00	41.75
02	+++	34.75	61.00
03	+++	40.75	73.50
04	++	64.00	83.25
05	++	51.25	75.50
06	+	47.00	54.50
07	+	40.00	51.00
SEmean		0.67	0.66
CD%		2.54	2.50

Table 2 Effect of BAP, ADS and NAA concentrations on bud breaking and shoot formation.

BAP	0.0	1.3	2.2	5.5	8.8	11.1	13.3	NAA
(µM)								
0.0	-	+	+	++	++	++ +	++ ++	
1.3	-	+	++	++	++	++ +	++ ++	
2.6	-	-	+	++	++ +	++ ++	++ ++	
5.4	-	-	-	++	++	++ ++	++ ++ +	
8.0	-	-	-	+	++	++ +	++ ++ +	
10.7	-	-	-	-	+	++ +	++ +	
16.1	-	-	-	-	-	+	++	
Adenine Sulphate (ADS)								
24.72	-	+	+	++	++ +	++ +	++ +	
61.87	-	+	++	++ +	++ ++	++ ++	++ ++ ++	

(-): No response; (+): Notable response, degree of response.

Table 3 Effect of BAP on Bud breaking percentage and shoot multiplication with respect to time.

BAP	Bud Breaking (%)	Bud Breaking (%)	Bud Breaking (%)
(µM)	(2 –weeks)	(2 –weeks)	(2 –weeks)
1.11	11.00	20.75	51.00
2.22	15.50	34.00	60.50
4.44	21.00	34.00	64.00
5.55	25.50	50.50	65.50
11.1	35.50	61.00	71.00
13.3	41.00	64.50	74.50
SEmean	0.473	0.55	0.485
CD1%	2.425	2.75	2.42

NAA (µM)	0.0	1.3	2.6	5.4	8.8	10.7	16.1	BAP
0.0	-	+	+	++	++	+++	+++	
1.1	+	+++	+	+	++	+++	+++	
2.2	+	+	∞∞∞∞∞	+	++	+++	+++	
4.4	+	+	++	∞∞∞∞∞	++	+++	+++	
5.6	++	++	++	++	++	∞∞	+++	
8.8	++	++	++	++	+++	∞∞	+++	

Table 4 – Effect of NAA and BAP on the callus formation
(-): No response; (+): Notable response, degree of response.

Table 5 Effects of (NAA +BAP) combinations of phytohormones on the formation of roots.

NAA+BAP (µM)	(%) Root initiation (4 weeks)	(%) Root initiation (6 weeks)	Number of Roots per Explant (4 weeks)	Number of Roots per Explant (6 weeks)
2.6+1.11	30.75	55.50	3.50	5.25
5.3+ 2.22	40.50	60.75	4.50	5.75
8.0+2.2	51.00	65.00	5.50	7.00
8.0+4.44	51.50	65.50	5.75	7.50
10.7+2.22	60.75	70.50	6.75	7.00
10.7+4.44	61.75	71.50	7.50	7.50
13.3+4.44	65.50	75.00	8.50	10.5
13.3+8.88	65.50	80.00	8.75	11.25
16.1+4.44	75.50	82.00	9.25	11.50
16.1+8.88	77.50	84.00	10.50	13.50
16.1+10.7	80.75	85.50	10.50	15.0
SE-mean	0.370	0.670	0.40	0.416
CD%	1.11	2.00	1.12	1.24

Fig :1 Mother plant of Stevia rebaudiana used was 1-2 months old with height of between 25 cm, and thickness of the stem was 2 cm. In-vitro propagation of Stevia rebaudiana the leaves and shoot tips were taken as the explants. The average width of leaf was 2 cm and the average length of the shoot tips were 2-3 cm obtained from a suitable location from the nursery of college of Bio-technology, B.A.U., Kanke, Ranchi.

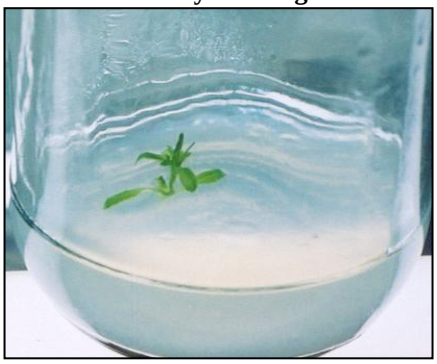

Fig: 2a Inoculation of shoot tip of Stevia rebaudiana in MS media having sterilized (with 0.1% $HgCl_2$) and left on moist filter paper in sterile petridishes to induce bud breaking. The procedure was done aseptically on laminar air flow bench.

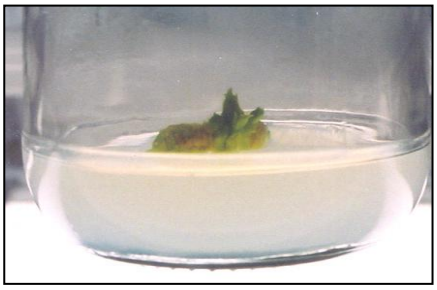

Fig:2b Shoot tip of Stevia rebaudiana showing bud breaking after two week in BAP(13.3 µM) +NAA(10.7 µM) in MS medium.

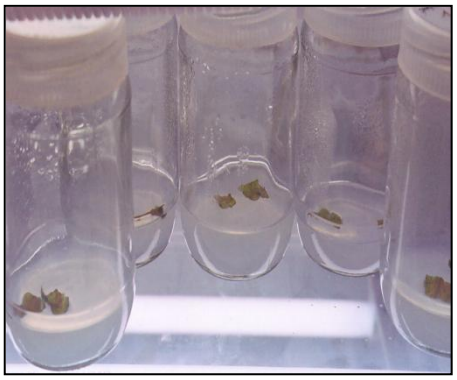

Fig:3 Shoot tip of Stevia rebaudiana showing shoot multiplication after six week in BAP (13.3 µM) +NAA (10.7 µM).

Fig:4 a & b Leaves of Stevia rebaudiana showing callus formation after six week in BAP of (13.3 µM) and NAA of (10.7 µM).

Fig:5 Shoot let of Stevia showing root initiation after four weeks of inoculation in addition of phytohormones with NAA1 (16.1 µM) + BAP (10.7 µM) in root medium .

Fig:6 Tissue cultured plant transfer into coca peat polybag in the mist chamber.

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