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MICROPROPAGATION OF STEVIA PLANT FROM NODAL SEGMENTS

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ABSTRACT

The experiment was conducted to develop and establish a reproducible protocol for plantlet regeneration in Stevia. Nodal cutting explants were cultured in Murashige and Skoog (MS) medium supplemented with different concentrations and combinations of α-Napthaleneacetic acid (NAA) and 6-Benzylamino purine (BAP). The combination of NAA at 1.0 mgl^{-1} and BAP at 1.0 mgl^{-1} resulted in the highest percentage (100%) of callus initiation. The maximum shoot regeneration and development of shoot was observed at the same combination. The developed shoots from nodal cuttings, upon transfer to the MS medium containing indole butyric acid (IBA) at 0.1mgl^{-1} resulted in best rooting within 8 days.

Key words : Micropropagation, plantlet regeneration, Stevia, Nodal segment

INTRODUCTION

Stevia rebaudiana Bert. belonging to the family Compositae, is one of the most valuable tropical medicinal plant. Stevia is originally a South Amarican wild plant (Katayma *et al*., 1976). But it could be found in semi-arid habitat ranging from grassland to scrub forest to mountain terrain. 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 (Bhole, 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). 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 is used as a table top sweetener, in soft drink, fruit juices, ice creams, yoghurts, sherbets, pastries, pies, baking, jams, sauces, pickles, jellies, desserts, chewing gum, candies, confectionary goods, sea- foods, vegetables, etc.

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 methods for rapid multiplication of Stevia. Seed germination of Stevia is often poor and less productive. Due to instability of plantlet produced through stem cutting,

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micropropagation method may overcome many of the limitations associated with conventional method and can be used for rapid multiplication. On the above mentioned perspective, the present study was undertaken to develop and establish a reproducible protocol for plantlet regeneration and to determine the suitable concentration.

MATERIALS AND METHODS

An experiment on *in vitro* callus induction and subsequent plantlet regeneration of Stevia was conducted at the Laboratory of the Department of Biotechnology, Bangladesh Agricultural University, Mymensingh in order to fulfill the objectives set in this research programme. Murashige and Skoog (MS) medium was used. 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. The nodal 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 0.5% mercuric chloride solution for 5 minutes under aseptic condition followed by washing 5-6 times with sterilized distilled water.

Explants were cultured on MS medium supplemented with different concentrations and combinations of BAP at (0, 1, 2.5 and 5 mgl^{-1}) and NAA at (0, 1, 2.5 and 5 mgl^{-1}) for callus initiation and proliferation. Calli from nodes were subcultured on the same medium. The calli were again cultured on MS medium supplemented with different concentrations and combinations of BAP at (0, 1, 2.5, 5 and 10 mgl^{-1}) and NAA at (0 & 1 mgl^{-1}) for shoot proliferation. Media supplemented with different concentrations of IBA (0, 0.1, 0.5 and 1.0 mgl^{-1}) and NAA (0, 0.1, 0.5 and 1.0 mgl^{-1}) were used for root induction. Culture vessels were incubated at 25 ± 1^0 C with 16 h photoperiod with light intensity of approximately 2000 lux.

RESULTS AND DISCUSSION

Callus induction

Induction of callus is an important factor for enhancing the rate of cell division resulting more fresh weight of callus. The cultured nodal segments were found to be able to produce profuse calli. The results of callus production and multiplication are presented in Table 1. Node explants upon culture on MS medium containing BAP plus NAA started callus initiation after 10 days (Fig. 1, A). The greatest amount of callus was obtained from node explants on the medium containing 1.0 mgl^{-1} NAA plus 1.0 mgl^{-1} BAP and callus weight was also highest (0.78 g). The other combinations showed poor performance and no callus was initiated on medium without growth regulators. Himanshu *et al*. (2006) observed best calli formation in MS medium containing 10 µM α-Napthaleneacetic acid plus 8.8 µM benzyl adenine. The friable and loose calli (brownish colour) did not show

shoot regeneration. Compact calli were considered as best for its high regeneration capacity. The compact calli were usually brownish to light green in colour (Fig. 1, B).

Table 1. Effects of different concentrations and combinations of NAA and BAP on callus initiation

Growth regulators (mg l^{-1})		% calli induction	Days to calli induction	Colour of calli	Callus morphologies	weight (g) of individual callus after 45 days
NAA	BAP					
0.0	0.0	0.00	0.00	-	-	0.00
0.0	1.0	0.00	0.00	-	-	0.00
0.0	2.5	40.00	16.00	Brownish	Friable	0.42
0.0	5.0	40.00	17.00	Brownish to Blackish	Friable	0.43
1.0	0.0	95.00	14.00	Brownish to Light green	Compact	0.75
1.0	1.0	100.00	13.00	Brownish to Light green	Compact	0.78
1.0	2.5	65.00	15.00	Brownish to Light green	Compact	0.50
1.0	5.0	55.00	16.00	Brownish to Light green	Compact	0.47
2.5	0.0	80.00	14.00	Brownish to Light green	Compact	0.74
2.5	1.0	90.00	14.00	Brownish to Light green	Compact	0.78
2.5	2.5	60.00	16.00	Brownish to Light green	Compact	0.52
2.5	5.0	50.00	17.00	Brownish	Friable	0.51
5.0	0.0	60.00	16.00	Brownish to Light green	Compact	0.50
5.0	1.0	65.00	16.00	Brownish to Light green	Compact	0.51
5.0	2.5	55.00	17.00	Brownish	Friable	0.51
5.0	5.0	50.00	17.00	Brownish	Friable	0.47
LSD		11.41	0.895	-	-	0.014

Plantlet regeneration

Cultured node explants produced calli on MS medium containing BAP and NAA. The calli began to increase in size and proliferation was started for new shoot at 13 days after subculture. The combination of NAA at 1.0 mgl^{-1} and BAP at 1.0 mgl^{-1} showed better performance and besides these the other concentrations and combinations showed poor performance. Node explants induced the highest percentage (87.50%) of shoots at minimum number of days (13) with NAA at 1.0 mgl^{-1} and 1.0 mgl^{-1} (Fig. 1, D). Single concentration of BAP (10.0 mgl^{-1}) acted as a growth promoter and produced higher number of shoots (12) $callus^{-1}$ with shorter shoot length (6.63 cm) but when BAP was combined with NAA (1.0 mgl^{-1}+ 10.0 mgl^{-1}) the number of shoots $callus^{-1}$ was reduced to (2.25) and shoot length increased (11.18 cm) (Table 2). Higher number of healthy shoots (46.9) obtained from nodal segments with 5.0 mgl^{-1} IBA and 2.0 mgl^{-1} NAA (Maharik and Gengaihi, 2003).

After inoculation of 28 days, shoots from node explants were isolated from the culture vessels and transferred to MS medium containing different levels of auxin (IBA) or (NAA) for rooting. The highest number of roots (95%) was induced on medium containing 0.1 mgl^{-1} IBA within the minimum number of days (8.00) (Fig. 1, E). Similar result was also obtained from 1.0 mgl^{-1} NAA (Table 3). But maximum number of roots (6.25) was obtained from node explants with 1.0 mgl^{-1} NAA. Himanshu *et al*. (2006) showed significant enhancement in the induction of rooting from 16.1 µM + 10.7 µM BA. The lowest concentration of IBA (0.1 mgl^{-1}) and the highest concentration of NAA (1.0 mgl^{-1}) gave similar results. So, it can be suggested that application of 0.1 mgl^{-1} IBA is much better than others. After rooting both shoots and roots continued to grow until complete well developed plantlets were established.

Table 2. Effects of different concentrations and combinations of BAP and NAA on shoot regeneration

Concentrations of growth regulators (mg l^{-1})		Percentage of shoot regeneration	Days to shoot induction	Number of shoots/callus	Shoot length (cm) after 28 days
NAA	BAP				
0.0	0.0	0.00d	0.00c	0.00f	0.00f
0.0	2.5	20.00c	15.00a	2.00e	7.50d
0.0	5.0	85.00a	13.25b	8.00b	7.30de
0.0	10.0	87.50a	13.25b	12.00a	6.63e
1.0	1.0	87.50a	13.00b	5.00c	10.50ab
1.0	2.5	75.00b	14.75a	4.50c	9.70c
1.0	5.0	72.50b	15.00a	3.50d	10.38bc
1.0	10.0	70.00b	15.25a	2.25e	11.18a
LSD		6.141	0.7886	0.7736	0.6969

Mean followed by different letter(s) in each column are significantly different at 5% level according to DMRT

Table 3. Effects of IBA and NAA on rooting

Concentrations of growth regulators (mgl^{-1})		Percentage of root induction	Days to root induction	Number of roots/plantlet
IBA	0.0	30.00d	11.25c	2.50cd
	0.1	95.00a	8.00d	5.00b
	0.5	50.00bc	13.50b	2.50cd
	1.0	45.00cd	14.50ab	1.50d
NAA	0.0	30.00d	11.25c	2.50cd
	0.1	60.00bc	14.50ab	2.50cd
	0.5	65.00b	14.75a	3.00c
	1.0	95.00a	8.00d	6.25a
LSD		16.98	1.040	1.052

Mean followed by different letter(s) in each column are significantly different at 5% level according to DMRT

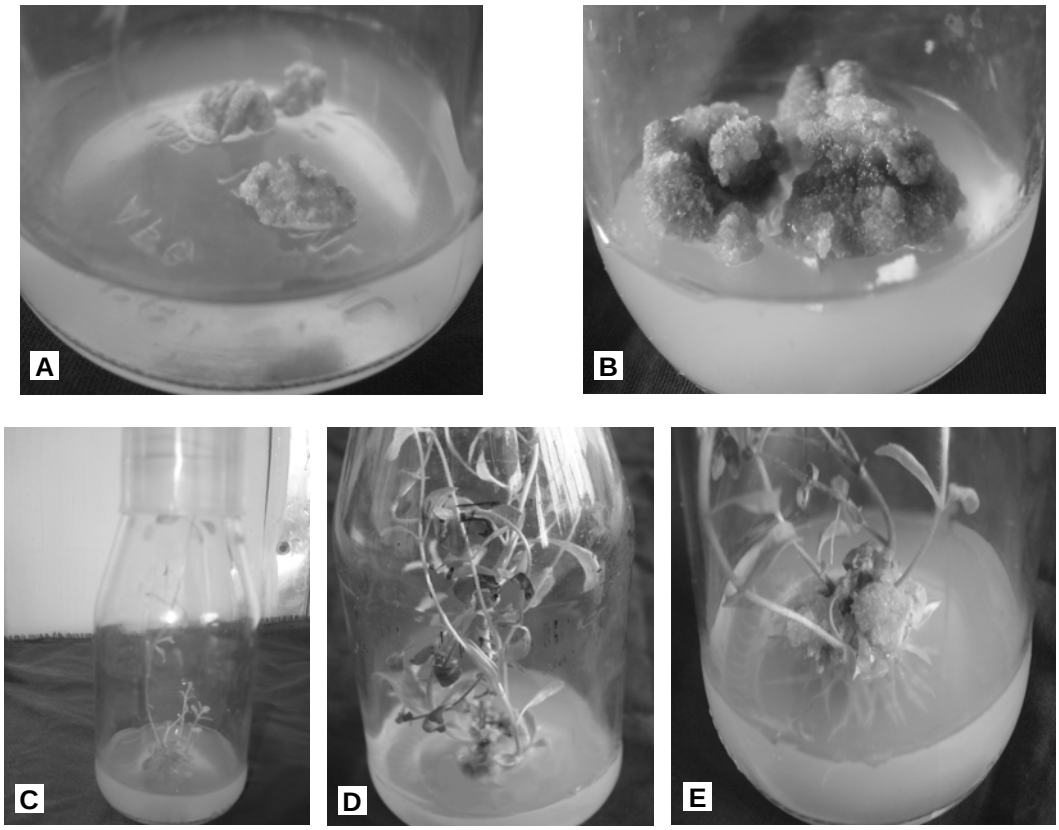

Fig. 1. Plantlet regeneration from nodal explants of Stevia
A) Callus from nodes at 16 days after inoculation;
B) Callus from nodes at 45 days after inoculation;
C) Shoot from callus at 14 days after inoculation;
D) Shoot from callus at 28 days after inoculation; and
E) Roots produced from shoots at 14 days after subculture.

Finally, it may be concluded that maximum calli induction, shoot regeneration were achieved with the combination of NAA at 1.0 mgl^{-1} and BAP at 1.0 mgl^{-1}. Shoots from node explants produced maximum number of roots containing 0.1 mgl^{-1} IBA in the medium. A protocol for the micropropagation Stevia has been developed in this study.

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