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IN VITRO REGENERATION STUDIES IN STEVIA THROUGH NODAL SEGMENT AND SHOOT TIP

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ABSTRACT

In vitro regeneration protocol was standardized for propagation of promising newly introduced non caloric sweetener and an antidiabetic medicinal plant *Stevia rebaudiana*. Nodal segment and shoot tip was used as an explants. Surface sterilization with $HgCl_2$ for 3 minutes gave best result of disinfection with maximum survival of explants for nodal segment and shoot tip also. For initiation MS + 1 mg L^{-1} BAP media was found best performing media. Maximum number of shoots per plant were observed on media MS + 2 mg L^{-1} BAP for both nodal segment and shoot tip explants.MS + 2 mg L^{-1} BAP media was found most promising media. Highest *in vitro* rooting (62.3 %) was obtained within 12.3 days on MSB5 media supplemented with1mg/l NAA. During hardening of rooted plants, 70% plantlet survival was observed on cocopeat in green house.

INTRODUCTION

Stevia rebaudiana Bertoni is an herbaceous perennial plant of the *Asteraceae* family. Stevioside, the major sweetener present in leaf and stem tissues of stevia, was first seriously considered as a sugar substitute in the early 1970s by a Japanese consortium formed for the purpose of commercializing stevioside and stevia extracts (Sharma *et al.*, 2013).

As demand of low carbohydrate sweetener is rising day by day. A good market is being developed for stevia domestically as well as internationally. Stevia leaves are 30 times sweeter than normal sugar. There are two compounds in stevia leaves, stevioside (10% -20%) and rebaudioside-A (1-3%). The extract of stevia rebaudioside-A is around 300-400 times sweeter than normal sugar and stevioside is stable at 100°C this is main advantage of Stevia over other sweetener (Hossain *et al.*, 2008).

Stevia having many therapeutic values as antihyperglycaemic, anticancerous and antihypersensitive properties (Rathore *et al.*, 2013). Being antihyperglycemic agent it stimulates the secretion of insulin from pancreas. Therefore, it is attractive natural sweetener to diabetic patients and others who are conscious about carbohydrate controlled diets (Uddin *et al.*, 2013).

Diabetes mellitus is a common disorder among the Indian population. It is estimated that diabetes would affect approximately 57 million people by the year 2025 (Gupta *et al.*, 2010). The management of diabetes is a global problem until now and successful treatment is not yet discovered.

The present study was undertaken to develop an efficient protocol for rapid *in vitro* propagation of *Stevia rebaudiana*.

MATERIALS AND METHODS

Stevia rebaudiana plants were collected from the Pune University, Pune, Maharashtra, India and maintained at State Level Biotechnology Centre, M.P.K.V., Rahuri (Maharashtra) during the year 2013-14.

Nodal segments containing one pair of auxiliary bud and shoot tip were used as explants for this study. Explants were washed in running tap water and then washed again thoroughly by adding a few drops of tween 20 to remove superficial dust particles as well as fungal and bacterial spores. Followed by, 3-4 washings with distilled water. Further operations were carried out in aseptic conditions. All the explants were surface sterilized with 0.1% and 0.05% mercuric chloride ($HgCl_2$) solution, 4% sodium hypochlorite (NaClO) for 1-5 minutes and immediately rinsed with sterile distilled water 3-4 times to remove all the traces of $HgCl_2$ and NaClO. For all the above studies, Murashige and Skoog(MS) Medium (1962) was used as sole basal medium. Plant growth regulator (PGR) stock solutions were freshly prepared for use. 3% of sucrose (30g/L, w/v), meso-inositol (100 mg/L, w/v) and required amount of plant growth regulators were added to the MS medium and the pH was adjusted to 5.8 with 1N HCl or 1N NaOH. The gelling agent, agar at 0.8% (w/v) was added to the prepared media and mixed well before dispensing into glassware. The contents were labelled and sterilized in an autoclave at 15 lb pressure for 15 minutes at 121°C. After sterilization, the medium was cooled to room temperature and stored in cool and dry place until used.

Sterilized explants were cut aseptically in laminar air flow cabinet and were implanted in MS medium fortified with different levels of cytokinins *viz*., benzylaminopurine (BAP), kinetin (Kin) and thidiazuron (TDZ) along with naphthalene acetic acid (NAA) to study the effect of different growth regulator combinations on establishment and multiple shoot induction. The inoculated cultures were incubated at 25 ± 2°C in an air conditioned culture room. Photoperiod was maintained 16 hrs (3000-3500 lux) supplied by cool white fluorescent tube lights daily followed by 8 hrs of darkness. Shoots of 2-3 cm height or with 4-5 leaves were transferred for root induction. The root organogenesis was observed on half MS medium and full strength MS alone and medium fortified with different levels of indole butyric acid (IBA) and NAA.

The experiment was carried out using Completely Randomized Design with ten treatments and ten replications. The observations were recorded in various experiments periodically from time to time. The data were compiled and subjected to statistical analysis and analysis of variance was done as per Panse and Sukhatme (1985).

RESULTS AND DISCUSSION

Stevia can be propagated by seed, by vegetative cutting and by tissue culture. Seed germination is very poor, commonly due to number infertile seed. Vegetative propagation is very low in number of individuals that can be obtained from single plant (Tiwari *et al*., 2000). The type and the concentration of the growth regulator in the medium were found to be important factor for multiple shoot induction (Bhat *et al.,* 2013, Pawar *et al.,* 2013, Pawar *et al.,* 2012,).

Sterilization of explants

In the present study, 0.05% $HgCl_2$, 0.1% $HgCl_2$ and 4% treatment of NaClO to explants for 1 to 5 minutes duration was tried for surface sterilization of nodal segment and shoot tip. The treatment of 3 minutes duration with 0.05 % $HgCl_2$ resulted best due to null contamination and survival of explants is maximum than further treatments. The 5 minute treatment also found effective to avoid contamination but death of explants was increased.

The treatments with 4% NaClO for 1 to 4 minutes duration for nodal segment were found ineffective due to high contamination rates and for shoot tip 1-3 minutes was ineffective due to contamination. Arya *et al*. (2001) reported that surface sterilization of nodal explants with 0.05 % $HgCl_2$ for 3-5 minutes duration proved best. Anbazhagan *et al*. (2010) reported that surface sterilization with freshly prepared 0.05% (w/v) mercuric chloride solution for 3-4 minutes and then given a dip in absolute alcohol was the best treatment for Stevia (Table 1)

Micro propagation

Few possible methods are available for rapid *in vitro* multiplication of propagules like meristem culture, shoot tip culture, axillary bud culture, somatic organogenesis and somatic embryogenesis (Murashige, 1974). The nodal segment and shoot tip culture are important among the different methods used in micropropagation study which are attempted in the present study.

Shoot differentiation

Direct regeneration of shoots, roots and whole plant without intervention of callus was observed from both shoot tip and nodal segment on basal MS medium. The regeneration of shoot tip and nodal segment were achieved on basal MS medium containing BAP along with Kin, TDZ, and NAA at different concentration (Table 2).

According to Bahurpe *et al*. (2013), Kumari and Pande (2011), Kopp and Nataraja (1990) not only cytokinin or auxin is responsible of *in vitro* regeneration but their concentrations and combinations are important.

Table1: Different treatments tried for surface sterilization of shoot tip and nodal segment explants

Disinfectant Duration	$HgCl_2$ (0.05%) Contamination%		$HgCl_2$ (0.1%) Contamination%		NaClO (4%) Contamination%	
	Shoot tip	Nodal segment	Shoot tip	Nodal segment	Shoot tip	Nodal segment
1	20	25	30	30	30	35
2	10	15	15	20	20	25
3	0	0	5	10	10	10
4	0	0	0	0	0	5

Table 2: Effect of media on days to shoot initiation

Tr.	Media composition	Nodal Segment	Shoot tip
T_1	MS + 1 mg L^{-1} BAP	7.6	8
T_2	MS + 1.5 mg L^{-1} BAP	9	9.6
T_3	MS + 2 mg L^{-1} BAP	13.3	11
T_4	MS + 1 mg L^{-1}BAP + 0.5 mg L^{-1} Kin	9.6	12
T_5	MS + 1.5 mg L^{-1}BAP + 0.5 mg L^{-1} Kin	12.6	114
T_6	MS + 2 mg L^{-1}BAP +0.5 mg L^{-1} Kin	15	15.6
T_7	MS + 1 mg L^{-1}BAP + 0.1 mg L^{-1} NAA	11.3	14
T_8	MS + 1.5 mg L^{-1}BAP + 0.2 mg L^{-1}NAA	14.3	16
T_9	MS + 2 mg L^{-1}BAP +0.5 mg L^{-1} NAA	14.3	17
T_{10}	MS + 2 mg L^{-1} BAP + 1mg L^{-1} TDZ	15.6	18.6
Mean		12.26	13.60
C.D.		1.87	3.81

For establishment of explants used in present study, they were cultured on MS medium supplemented with different concentrations of cytokinin (BAP, Kin and TDZ), and auxin (NAA).

Days to shoot initiation

The number of days required for shoot initiation in two different explants varied significantly. There was a significant difference within the medium, for all observations. The most early shoot initiation was observed in 7.6 days from nodal segment on T_1 (MS + 1 mg L^{-1} BAP) medium. While earliest shoot initiation from shoot tip explant, was recorded on same medium in 8 days.

Table 3: Effect of media on percent shoot multiplication

Tr.	Media composition	Nodal segment	Shoot tip
T1	MS + 1 mg L^{-1} BAP	70.3	50.3
T2	MS + 1.5 mg L^{-1} BAP	77	55
T3	MS + 2 mg L^{-1} BAP	85.6	62.6
T4	MS + 1 mg L^{-1} BAP + 0.5 mg L^{-1} Kin	72	54
T5	MS + 1.5 mg L^{-1} BAP + 0.5 mg L^{-1} Kin	68.5	53.3
T6	MS + 2 mg L^{-1} BAP + 0.5 mg L^{-1} Kin	80.3	48.6
T7	MS + 1 mg L^{-1} BAP + 0.1 mg L^{-1} NAA	62.4	42
T8	MS + 1.5 mg L^{-1} BAP + 0.2 mg L^{-1} NAA	70.6	36
T9	MS + 2 mg L^{-1} BAP + 0.5 mg L^{-1} NAA	60.6	32.3
T1	MS + 2 mg L^{-1} BAP + 1mg L^{-1} TDZ	68.3	38.3
Mean		71.13	47.63
CD		6.08	4.68

Table 4: Effect of media on number of shoots per explant

Tr.	Media composition	Nodal Segment	Shoot tip
T_1	MS + 1 mg L^{-1} BAP	4.6	4.6
T_2	MS + 1.5 mg L^{-1} BAP	7	5
T_3	MS + 2 mg L^{-1} BAP	8	6
T_4	MS + 1 mg L^{-1} BAP + 0.5 mg L^{-1} Kin	4.3	5.3
T_5	MS + 1.5 mg L^{-1} BAP + 0.5 mg L^{-1} Kin	6.3	3
T_6	MS + 2 mg L^{-1} BAP +0.5 mg L^{-1} Kin	2	2.6
T_7	MS + 1 mg L^{-1} BAP + 0.1 mg L^{-1} NAA	3.6	4.6
T_8	MS + 1.5 mg L^{-1} BAP + 0.2 mg L^{-1} NAA	3	3.6
T_9	MS + 2 mg L^{-1} BAP +0.5 mg L^{-1} NAA	2	2.6
T_{10}	MS + 2 mg L^{-1} BAP + 1mg L^{-1} TDZ	3.6	3.3
Mean		4.33	4.30
C.D.		1.98	2.50

Table 5: Effect of culture medium on in vitro rooting

Tr.	Media composition	Days to Rooting	Rooting %
T_1	1/2MS	-	-
T_2	MS	-	-
T_3	MS + 1 mg L^{-1}NAA	12.3	62.3
T_4	MS + 1.5mg L^{-1}NAA	14	55
T_5	MS + 2 mg L^{-1}NAA	15.6	42
T_6	MS + 1 mg L^{-1} IBA	13.3	52.3
T_7	MS + 1.5 mg L^{-1} IBA	15	48.6
T_8	MS + 2 mg L^{-1} IBA	17.6	51.2
T_9	1/2MS + 1 mg L^{-1} NAA	16	39.9
T_{10}	1/2MS + 1 mg L^{-1} IBA	18.3	36.3
Mean		12.23	38.87
C.D.		2.28	5.67

Percent shoot multiplication

Percent shoot multiplication obtained ranged from 60.6 to 85.6 percent for nodal segment and 32.3 to 62.6 percent for shoot tip explants. There was a significant difference within the treatments, for all observations. Highest percent of response for multiplication was observed in nodal explants showing 85.6 percent multiplication on the medium T_3 (MS + 2 mg L^{-1} BAP), while the lowest percent of response (60.6 percent) on T_9 (MS + 2 mg L^{-1} BAP + 0.5 mg L^{-1} NAA) media (Table 3). Similar results were observed in micropropogation of Kinnow through nodal segment by Sharme *et al*. (2012).

Best response for multiple shoot induction in shoot tip explant was observed on T_3 (MS + 2 mg L^{-1} BAP). While the lowest percent of response was observed on T_9 (MS + 2 mg L^{-1} BAP + 0.5 mg L^{-1} NAA)

Number of shoots per plant

The maximum number of shoots per explants (eight) was obtained from nodal explants as compared to shoot tip (six) on the medium i.e. on T_3 (MS + 2 mg L^{-1} BAP). In the present study, gradual increase in shooting frequency, number of shoots per explant was observed with increasing concentrations of BAP (Table 4).

Rooting response

It was revealed from table 5 that, the MS medium without growth regulator and 1/2 MS medium was not useful for present study. T_3 (MS + 1 mg L^{-1} NAA) were found to be the best for rooting in terms of rooting percentage (62.3 %) and days to root initiation (12.3).

Late rooting (18.3 days) was observed on media T_{10} (1/2 MS + 1 mg L^{-1} IBA). Percent rooting was also low on same medium (36.3 %).

Hardening

The plants produced through micropropagation are very delicate to face ambient environmental conditions (Jagatheeswari and Ranganathan 2012).

In the present study, rooted plantlets were transferred from culture bottles to plastic cups containing cocopeat as media. Good percentage of survival (70%) in polyhouse was observed. The survived plantlets were hardened in shade net for future planting in field.

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