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ROLE OF SUCROSE AND SEASON ON RAPID *IN VITRO* REGENERATION FOR TWO *STEVIA* GENOTYPES

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ABSTRACT
In order to develop a protocol for high efficiency *in vitro* regeneration of two cultivars of stevia ' CIM madhu' and 'CIM mithi' respectively, the influence of season on the rate of multiplication was investigated in shoot cultures on Murashige and Skoog (MS) media, supplemented with different combinations of growth regulators. *In vitro* performance of explants indicated a positive correlation between shoot proliferation and season in both genotypes of *stevia*. The mean number of shoots formed per explant was higher when 'CIM madhu' and 'CIM mithi' cultivars were cultured on MS media with 4% sucrose and Organics (MS)x2 , in the active growing season. In both cultivars, the mean number of shoots formed per explant was slightly higher when sub-cultured on MS medium, in the month of January, April and August, which were proven to be the most effective season.

KEYWORDS: Growth regulators, leaf segment, sucrose, vitamins.

INTRODUCTION

Stevia rebaudiana Bert. the most valuable tropical medicinal plant, a member of family Asteracecae (Aamir *et al.,*, 2010). It is commonly known as *stevia*, honey leaf, ya wan, sweet grass (Inamake *et al.,* 2010). It is the only natural sweetener in the world. it is a small herbaceous plant with carbohydrate-based compounds mainly steviosides that are 200 to 300 times sweeter than cane sugar. *Stevia* plants are a good source of carbohydrates (61.93% d.w.), protein (11.41% d.w.), crude fiber (15.52% d.w.), minerals. Eight of *stevia* glycosides were discovered, viz. Dulcosides A, rebaudiosides A-E, steviobioside and stevioside (Khoda *et al.,* 1976; Kinghorn *et al.,* 1984). Diet conscious and diabetic persons with hyperglycemia can use steviosides as an alternative sweetener (Din *et al.*, 2006). The medicinal uses of *stevia* are as a natural sweetener for diabetics, treating, obesity, hyperactivity, hypertension, carbohydrate cravings, tobacco and alcohol cravings, hypoglycaemia, indigestion, yeast infections, skin toning and healing heart disease, and dental maladies (Kinghorn and Soejarto, 1985) anti-tumor (Yasukawa *et al.,* 2002) antihypertensive (Ferri *et al.,* 2006).

The seeds of *S. rebaudiana* show a very low germination, it is generally multiplied vegetatively by grafting. Micropropagation method is especially applicable to species in which clonal propagation is needed (Gamborg and Phillips, 1995). Since the rapid clonal multiplication of plants *in vitro* is quicker and cheaper than *in vivo.* Due to the above-mentioned difficulties, tissue culture is the only alternative method for rapid propagation of *S. rebaudiana* plants.

MATERIALS AND METHODS

This research experiment was conducted at the Central Tissue Cultural Laboratory, Indian Agricultural Research Institute, New Delhi.-110012, India. The *in vitro* regenerated leaf segments were used as explant to investigate the effects of different concentrations of sucrose (3% to 6%) and organic additives interaction on growth and development on subsequent plantlets regeneration of *Stevia* cultivars. Stock solutions of all components were prepared with appropriate amount of all the components. All stock solutions were mixed properly. Modified concentration of MS salts and standard concentrations of MS salts were used. The pH of media was adjusted to 5.74 to 5.8. About 15 ml of the medium were dispensed in each culture tube before autoclaving at 121 ºC for 15 min under pressure of 15 Psi, the cultured tubes were sealed with cotton plugs. The *in vitro* regenerated leaf segments were inoculated upon MS media (Murashige and Skoog, 1962) with an addition of cytokinins and auxins as per the protocol described by Verma *et al.,* (2011). After culturing the explants, incubated in the growth room under 2000 Lux light at 25$\pm$ 2°C with 16 hour light and 9 hour dark period in every 24 hour cycle. The experiment was laid out in Completely Randomized Design (CRD). The data were collected and recorded at 15 days interval. Each experiment was performed in triplicates.

RESULT AND DISCUSSION

Stevia is an important medicinal plant and present study was conducted to optimize the effects of different level of sucrose, organic additives and season interaction on growth and development of plantlets regeneration of two cultivars of *stevia*. Sucrose has been used as a major carbohydrate source in the induction medium. It is the main source of energy for *in vitro* plant tissue cultures as these have insufficient autotrophic ability. Sucrose not only acts as an external energy source but also help to maintain osmotic potential of the culture (Nowak *et al.,*,

2004; Siwach *et al.*,, 2011) which would permit the absorption of mineral nutrient present in medium, essential for optimal proliferation. Maximum organogenesis frequency (97.80 % and 96.80%) was obtained in medium having 4% sucrose (Table 1), with respect of minimum days 15.22 and 16.22 days taken for microshoots regeneration in CIM madhu and CIM mithi respectively. Minimum number of microshoots (3.80) par calli was reported at 3%. Highest number of microshoots (15.09) was obtained in medium having 4% sucrose. The frequency declined considerably at higher concentration of sucrose. These findings are consistent with the previous reports (Reinert and Bajaj, 1977, Chen, 1978; Chen *et al.,* 1991, Sandhu *et al.,* 1993, Pande and Bhojwani, 1999). In rice, higher level of sucrose besides promoting the induction and growth of callus is also useful in organogenesis (Chi-Chang-Chen, 1978). Wang *et al.,* (1977) reported that sucrose concentration of above 6% in the induction medium increased proportion of albino plants. Reinert *et al.,* (1977) suggested that sucrose level of 2 - 5% is good for rice anther culture. Shahnewaz1 and Bari(2004) used N6 medium containing 0, 1, 2, 3, 4, 5 and 6% sucrose for regeneration for anther culture of rice and found 4% of sucrose most suitable. Naik and Nayak(2005) used various concentration of sucrose 30,60 and 90 mg/l for *in vitro* bulblet production in *Ornithogalum virens* and found 60 mg/l of sucrose most effective. A significant effect of carbon source concentration has been reported by Gracia *et al.,* (2002); Siwach *et al.,* (2011) in *Olive* and *Ficus religiosa* L. respectively.

TABLE-1: Effect of carbon source on shoot regeneration on callus derived from leaf explants

Treatments	Regeneration (%)		Days to shoot initiation		No. of microshoots /calli	
Sucrose	CIM madhu	CIM mithi	CIM madhu	CIM mithi	CIM madhu	CIM mithi
3%	24.98	22.65	32.97	33.64	3.80	3.80
4%	97.80	96.80	15.22	16.22	15.26	14.91
5%	66.05	65.05	21.14	21.81	8.39	8.16
6%	46.27	44.27	23.10	24.10	6.89	6.56
Mean	58.77	57.19	23.11	23.94	8.52	8.36
CD at 5 % for						
Crop (A)	0.85		0.61		N.S.	
Treatment (B)	1.21		0.87		0.71	
AXB	N.S.		N.S.		N.S.	

Between various levels of MS organics tested for shoot proliferation, modified concentration of organics (MS)x2.5 was found more effective than standard and other modified levels (Table 2). Shoot formation was evaluated in terms of average shoot length (cm) and shoot number per explant and average shoot length. Organics (MS) x2.5 gave maximum number of shoots (16.85 and 15.63) in CIM madhu and CIM mithi respectively. Maximum mean length of (8.62cm and 8.32cm) was achieved on the same level of organics (MS) x2.5 respectively. These four vitamins; myo-inositol, thiamine, nicotinic acid, and pyridoxine are ingredients of Murashige and Skoog (1962) medium and have been used in varying proportions for the culture of tissues of many plant species.

TABLE-2: Effect of modification of micro and organics constituents on shoot proliferation in two *stevia* genotypes

Treatments	No. of shoots		Shoot length (cm)	
	CIM madhu	CIM mithi	CIM madhu	CIM mithi
Organics (MS)x1	6.52	6.00	3.54	3.31
Organics (MS)x1.5	9.94	9.43	5.54	5.10
Organics (MS)x2	12.18	11.18	6.74	6.22
Organics (MS)x2.5	16.85	15.63	8.62	8.32
Mean	11.37	10.56	6.11	5.74
CD at 5 % for				
Crop (A)	0.47		0.28	
Treatment (B)	0.67		0.40	
AXB	N.S.		N.S.	

The influence of season on the various growth factors was also investigated. The effect of season on culture establishment is showing in (Fig. 1). Maximum number of shoots (11.60 and 12.26) was proliferated in the month of October followed by September, February and March respectively, with respect to minimum days (14.70) taken for shoot emergence and number of shoots per culture (Table 3). Maximum culture (91.98% and 92.80%) was achieved on the month of October with respect to minimum days. There was a gradual reduction in culture establishment in the month of January (24.95 and 28.61%) in CIM madhu and CIM mithi respectively. Roy *et al.,*

(2004) observed best response in shoot regeneration and multiplication, when the culture was initiated during the winter season. Seasonal variation of *in vitro* shoot induction was also reported in many other plant materials (Zaman *et al.,* 1996). Sutan and Isca (2010) also investigate the influence of season on micropropagation in *Fragaria* and *Potentilla* two intergenic varieties. He observed significant variation in response to seasons among both the varieties. Verma *et al.,* (2011) also studied the seasonal effect on bud break in *stevia*

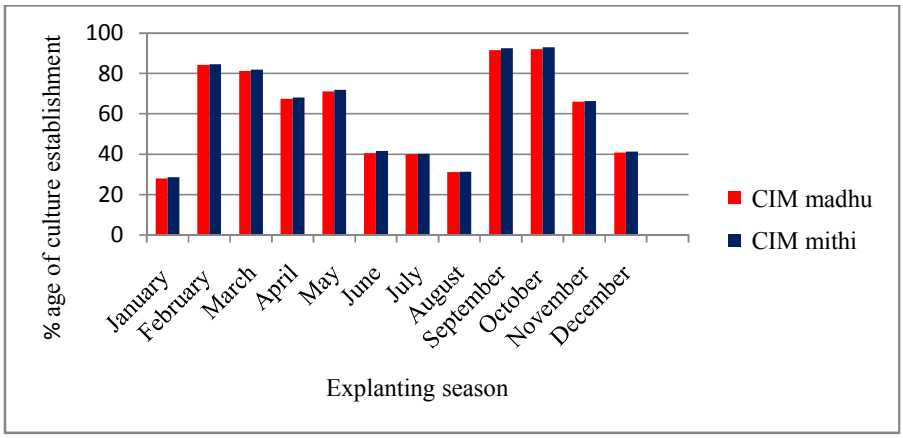

FIGURE 1. Effect of season on culture establishment in two *stevia* genotypes

TABLE-3: The influence of season on time taken for shoot emergence and shoot proliferation in two *stevia* cultivars.

Treatments	Days to shoot emergence		No. of shoots	
	CIM madhu	CIM mithi	CIM madhu	CIM mithi
January	33.44	32.77	2.33	2.40
February	21.40	21.70	4.16	4.33
March	24.73	24.07	4.17	4.17
April	28.79	27.29	3.36	3.43
May	23.69	22.69	4.67	4.75
June	26.91	26.24	4.13	4.26
July	26.36	25.69	4.22	4.29
August	27.70	27.04	2.58	2.68
September	16.32	16.17	10.49	11.16
October	15.03	14.37	11.60	12.26
November	19.12	18.92	7.01	8.11
December	20.31	19.64	6.09	6.36
Mean	23.65	23.09	5.40	5.72
CD at 5 % for				
Crop (A)	0.39		0.28	
Treatment (B)	0.96		0.70	
AXB	N.S.		N.S.	

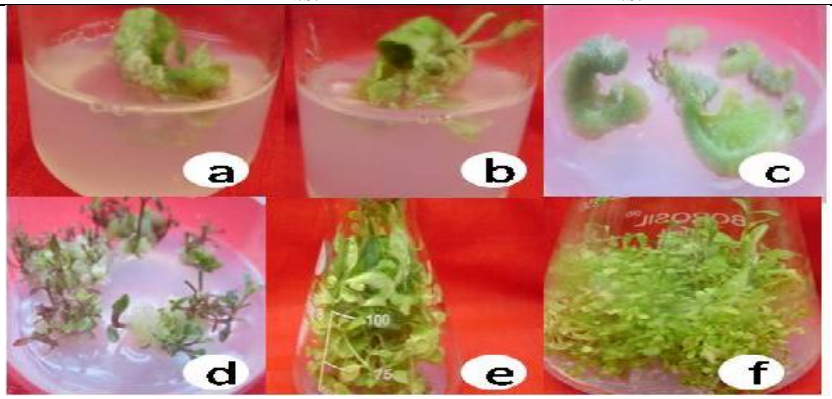

FIGURE 2. organogenesis and proliferation in *stevia*
a) Callus initiation; (b-d) Organogenesis; (e-f) Proliferation

CONCLUSION

We have established an efficient and rapid protocol for proliferation of callus culture initiated from *in vitro* regenerated leaf explant. Growth of culture showed variation of responses depending upon season, concentration of sucrose and strength of organic salt. It was resulted from this study that, January, April and

August are the most favouring month for callus induction and culture growth.

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