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Comparative Study on Callus Induction, Proliferation and Plantlets Regeneration In Two Cultivars of *Stevia rebaudiana* Bertoni – The Only Non Caloric Natural Sweetener

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ABSTRACT

The present report describes a comparative study for plant regeneration through callus morphogenesis in two different cultivars 'CIM madhu' and 'CIM mithi' of *S. rebaudiana*. The leaf explants were cultured on MS medium supplemented with IBA (1.0-4.0 mg/l) in combination with BAP (0.2 mg/l) for callus induction. Among the various tested combinations for shoot regeneration, maximum multiplication was recorded with MS + Kn (2.0 mg/l) + NAA (0.2 mg/l) + ADS (40 mg/l). Half-strength MS medium with IBA (0.2 mg/l) + AC (100 mg/l) was the best medium for the *in vitro* rooting of regenerated shoots. A comparison of different hardening media was also studied between two cultivars. The micro-plantlets hardened in plastic pots filled with sand: soil: vermiculite (1:2:1), covered with transparent polythene bags took minimum time to glass house transfer with maximum survival rate. CIM-madhu showed good callus induction, proliferation and regeneration ability in comparison to CIM-mithi. In comparison, higher rooting percentage was obtained in CIM-madhu with 97 % survival rate. The ability of 'CIM-madhu' to induce callus and regenerate successful plantlets under these conditions suggests that this cultivar is moderately suitable for micropropagation purposes.

Keywords: Stevia rebaudiana, growth regulators, leaf segment, callus induction, plant regeneration

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INTRODUCTION

Stevia rebaudiana Bertoni (family Asteraceae) commonly known as "Sweet Weed", "Sweet Leaf", "Sweet Herbs" and "Honey Leaf", is the most valuable tropical natural sweetener perennial herb indigenous

to the Paraguay and Southern Brazil (Ali *et al.,* 2010; Singh *et al.,* 2011). Currently, stevia is commercially grown in Central America, Japan, South East Asia, Israel, China and Canada (Patel & Shah, 2009). Its leaves contain secondary metabolites such as stevioside, rebaudioside A., rebaudioside C. and dulcoside A. which produce a sweet taste having no caloric value (Kinghorn, 1987; Din *et al.,* 2006). So, it produces non-toxic, non-calorie, non-plaque, non-fermentative, flavour enhancing, non-carcinogenic and non-addictive sweetness absolutely safe for diabetics, phenyl ketonuria patients and diet conscious persons (Gregersen *et al.,* 2004). Its leaves also contain protein, fibres, carbohydrates, phosphorus, iron, calcium, potassium, sodium, magnesium, rutin, iron, zinc, vitamin A and vitamin C. It is used for different therapeutic effects in diabetes, obesity, hyperactivity, hypertension, carbohydrate cravings, tobacco and alcohol cravings, hypoglycaemia, indigestion, yeast infections, skin toning and healing (Yasukawa *et al.,* 2002; Lailerd *et al.,* 2004; Singh *et al.,* 2011; Verma *et al.,* 2011). It is also used in sweet sauces, pickles, bakery, beverages and confectionery sectors in Japan and Korea (Preethi *et al.,* 2011).

The seeds of *S. rebaudiana* show a very low germination percentage and do not produce uniform emergence, resulting in great variability in plant growth and maturity (Sivaram & Mukundan, 2003; Verma *et al.,* 2011). Vegetative propagation is too slow having the possibilities of pathogen attack on the tissues (Debnath, 2008; Mishra *et al.,* 2010). Therefore, *in vitro* plant culture techniques may be an effective alternative for propagation and conservation of plants of such an economic importance in which conventional methods show limitations (Yadav *et al.,* 2013a, 2013b).

Although attempts have been made by several workers for *in vitro* studies on *S. rebaudiana* using various physical and biological factors (Kornilova & Kalashnikova, 1997; Din *et al.,* 2006; Ahmed *et al.,* 2007; Ibrahim *et al.,* 2008; Patel & Shah, 2009; Satpathy & Das, 2010; Ali *et al.,* 2010; Singh *et al.,* 2011; Verma *et al.,* 2011), considerable efforts are still required to make it more economical and practical.

The genotype used and its interaction with the various physical and biological factors also influence the different stages of *in vitro* multiplication (George, 1993; Yadav & Singh, 2012; Yadav *et al.,* 2012). In the light of the above-referred importance and demand, the present investigation was performed in order to gain information on the comparative effects for *in vitro* suitability of two varieties (CIM- madhu and CIM- mithi) of *S. rebaudiana.*

MATERIAL AND METHODS

Collection of Plant Material

The experiment was carried out in the Central Tissue Culture Laboratory of Lal Bahadur Shastri Building IARI, New Delhi. Two varieties of *S. rebaudiana* (CIM-madhu and CIM- mithi) were collected from the nursery of Central Institute of Medicinal and Aromatic Plants (CIMAP) Lucknow (India) and maintained under glasshouse conditions. Leaf segments (1cm

x 1cm) excised from these two different varieties served as a source of explants for micropropagation.

Surface Sterilization

The explants were first washed with running tap water to remove dust particles, and then surface sterilized with Tween-20 (2 drops per 100 ml water) for 5 min, followed by washing under tap water to remove all the detergent traces. The sterilized explants were then treated with 0.1% (w/v) mercuric chloride for 3-5 min under aseptic conditions. These explants were thoroughly washed 4-5 times with autoclaved double distilled water to remove the traces of mercuric chloride. Excess water adhering on explants surface was removed using autoclaved blotting paper under a laminar air flow chamber.

Culture Condition

The explants were trimmed with sterilized blade and finally inoculated on MS (Murashige and Skoog, 1962) medium with different concentrations and combinations of growth regulators (BAP and IBA) containing 30 g/l sucrose and 8 g/l agar (Himedia, India) to initiate callus culture. The pH of all media was adjusted to 5.8 with 1 N NaOH or 1 N HCl and finally autoclaved at 1.05 kg/cm^{-2} at 121°C for 20 min. The cultures were maintained at a temperature of 25±2ºC with a 16/8 h light/ dark photoperiod under an illumination of 20 µmol m^{-2} s^{-1} photosynthetic photon flux intensity provided by cool- white fluorescent light.

Callus Induction

The calli formed from the leaf explants were periodically sub-cultured every four week for multiplication and maintenance on callus proliferation medium, MS nutrients supplemented with IBA (4.0 mg/l) + BAP (0.2 mg/l).

Indirect Shoot Regeneration

For shoot regeneration, the calli were further sub-cultured on shoot induction medium, MS salts fortified with different concentrations and combinations of Kn (0.5- 2.0 mg/l), BAP (0.5-2.0 mg/l), ADS (40-60 mg/l) and NAA (0.1- 0.2 mg/l). The best resulting medium formulation was identified in terms of response, number and length of shoots.

In vitro Rooting and Acclimatization

For *in vitro* root induction, individual shoots (2 cm long) were excised from the shoot clump and transferred to half-strength MS medium (3% sucrose and 0.8 % agar) with different concentrations of IBA (0.2-0.5 mg/l) alone and in combination with activated charcoal (AC). The cultures were maintained under the same conditions as for shoot induction. When adequate rooted shoots were obtained, the plantlets were carefully pulled out from the medium and kept under running tap water, using a fine brush to remove the medium sticking to the root system. These plantlets were then transferred to pots containing different substrates, viz. vermicompost: peat moss: sand (1:1:1) and sand: soil: vermicompost (1:2:1). The pots were covered with

polyethylene membranes to ensure about 80% relative humidity. The potted plants were irrigated with MS (half strength) salt solution devoid of sucrose and myo-inositol every 3 days. After about 4 weeks, these plants were transferred to larger pots and maintained under glasshouse conditions. Survival rate was assessed after 3 months.

Statistical Analysis

Complete Randomized Design (CRD) was used for the *in vitro* culture experiments. Each single explant was considered as an experimental unit. Each treatment consisted of 3 replicates.

RESULTS

The initiation of callus started from the cut ends of the explants in the beginning and gradually extended to all over the explant. The responses of the leaf explants varied with the growth regulator combinations. However, the nature of the response of different cultivars to a particular growth regulator combination was more or less similar. The control MS medium without any hormone was also capable of inducing callus but only in trace amount (Table 1).

The effects of different concentrations of IBA and BAP on callus induction of *S. Rebaudiana* are presented in Table 1. The increase of callus formation efficiency was observed with the increase of the IBA concentration. The best callus growth (in terms of biomass) was obtained when 4.0 mg/l IBA + 0.2 mg/l BAP was used in the medium. Friable, greenish yellow callus was observed irrespective of the genotypes (Fig.1a-1b).

The above mentioned calli derived from leaf explants were subcultured on MS medium supplemented in different concentrations and combinations of Kn (0.5 - 2.0 mg/l), BAP (0.5 - 2.0 mg/l), ADS (40-160 mg/l) and NAA (0.1 - 0.2 mg/l) for shoot regeneration (Table 2). After, 1-2 weeks of transfer to regeneration medium, the sub-cultured calli enlarged rapidly and started emerging out green shoot buds from the surface.

Among the various combinations, the presence of Kn was found to be more

TABLE 1
Effects of IBA and BAP on callus induction from leaf explants in two *stevia* genotypes (CIM-Madhu and CIM-Mithi). Data were recorded after 45 days of culture.

	Callusing %		Days of callus induction	
Treatments (mgL^{-1})	CIM-madhu	CIM-mithi	CIM-madhu	CIM-mithi
MS devoid of Hormones (control)	10.55	.44)	42.56	43.76
MS+IBA (1.0) + BAP (0.2)	66.26	56.67	30.92	32.16
MS+IBA (2.0) + BAP (0.2)	79.48	78.17	22.20	24.13
MS+IBA (3.0) + BAP (0.2)	93.62	92.32	18.08	18.44
MS+IBA (4.0) + BAP (0.2)	97.79	96.50	13.10	13.58

Results represent CD at 5% of three replicated experiments.
Whereas: Treatments (A) = 0.23; 0.23, Cultivars (B) = 0.36, 0.36, A x B = 0.51, 0.51

effective than BAP. The combination of Kn with NAA/ADS induced the highest number of shoot buds in the least number of days. Among the cultivars, CIM-madhu produced the highest number of shoot buds in less number of days in comparison to CIM-mithi (Table 2; Fig.1c-1d). Plantlets of *S. rebaudiana* longer than 2 cm were transferred into MS medium (half-strength) supplemented with IBA (0.2- 05 mg/l) and active charcoals (AC) to initiate roots. MS basal medium without any growth regulator showed a delayed and weak response (Table 3). The results showed 96 and 95% rooting rates in CIM madhu and CIM mithi in medium ½ MS + 0.2 mg/l IBA + 100 mg/l AC respectively (Fig.1e-1f, Fig.2).

The micro-plantlets hardened in plastic pots filled with vermicompost: soil: sand (1:2:1), covered with transparent polythene bags took minimum time (15 days) to glass house transfer and showed maximum survival rate (Fig.1g-1h; Table 4). It was observed that gradual acclimatization of *in vitro* grown plants to the external environment is most essential for s*tevia*.

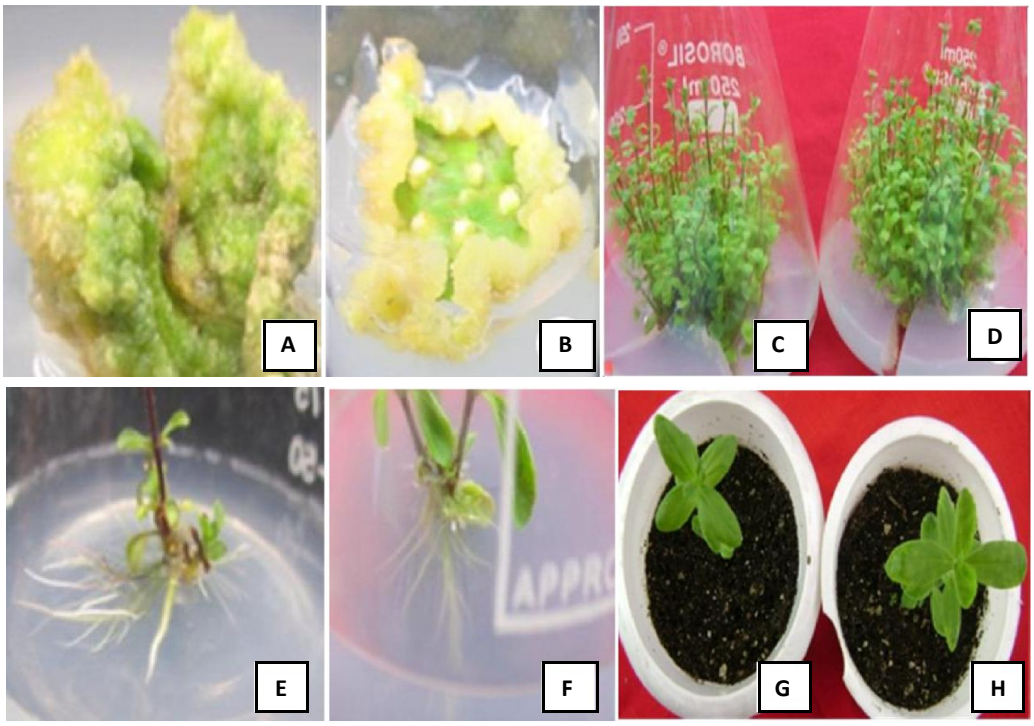

Fig.1: Callus morphogenesis and plant regeneration in *S. rebaudiana*. (A-B): Callusing on MS + IBA(4.0 mgL^{-1}) + BAP (0.2 mgL^{-1}); (C-D): organogensis on MS + Kn (2.0mgL^{-1}) + AS (40mgL^{-1}) + NAA (0.2mgL^{-1}); (E-F): Rooting on MS + IBA (0.2 mgL^{-1})+ AC (100mgL^{-1}); (G-H) hardening of plantlets in vermiculite: soil: sand (1:2:1).

TABLE 2
Effects of growth regulators on shoot regeneration on callus derived from leaf explants in two *stevia* genotypes. Data were recorded after 45 days of culture.

	CIM-madhu			CIM-mithi		
Treatments (mgL^{-1})	Regeneration percent	Days for shoot initiation	Number of microshoot /calli	Regeneration percent	Days for shoot initiation	Number of microshoot /calli
Control	8.00	41.68	1.22	6.02	43.11	1.00
MS+2.0BAP+0.2NAA	33.43	26.45	2.31	33.13	26.93	2.72
MS+1.0BAP+0.2NAA	39.76	28.13	2.05	38.55	30.22	2.16
MS+2.0 Kn+ 0.2NAA	69.04	16.78	7.16	67.33	20.13	8.51
MS+2.0Kn+0.2NAA+40AS	78.91	12.21	11.98	77.13	12.84	12.44
MS+1.0Kn+0.2 NAA+40AS	73.96	15.15	12.51	73.06	18.24	12.10
MS+0.5BAP+0.5Kn+0.1NAA	41.89	26.24	3.23	40.11	29.25	4.86
MS+1.0BAP+0.2NAA+40AS	66.78	21.22	6.98	64.37	24.87	7.87
MS+2.0BAP+0.2NAA+40AS	57.92	20.44	4.53	57.25	22.53	6.88

Note: Results represent CD at 5% of three replicated
Cultivars (A) = 0.18; 0.16; 0.09, Treatments (B) = 0.39; 0.35; 0.21; AXB = 0.56; 0.50; 0.29

TABLE 3
Effects of IBA and AC on number of roots per micro cuttings and mean length in two *stevia* genotypes CIM madhu and CIM mithi. Data were recorded after 30 days of culture.

	No. of roots/microcutting		Root length (cm)	
Treatments (mgL^{-1})	CIM-madhu	CIM-mithi	CIM-madhu	CIM-mithi
½ MS devoid of auxins	3.23	3.06	1.10	1.02
½ MS + 0.2 IBA	9.13	8.76	3.30	3.05
½ MS + 0.5 IBA	7.23	7.06	2.43	2.13
½ MS + 0.2 IBA + 100 mgL-1 AC	13.13	12.73	4.23	4.00
½ MS + 0.5 IBA + 100 mgL-1 AC	9.43	9.16	2.93	2.50

Results represent CD at 5% of three replicated experiments.
Treatment (A) = 0.07, 0.09; Cultivar (B) = 0.12, 0.14; A X B= N.S., N.S.

TABLE 4
Effects of hardening strategies on plantlet survival and time taken to glass house transfer.

Treatments (mgL^{-1})	CIM-madhu		CIM-mithi	
	Survival	Time taken to glass house transfer	Survival	Time taken to glass house transfer
Vermicompost: peat: moss: sand (1:1:1)	50.23	31.40	49.63	31.76
Vermicompost: soil: sand (1:2:1)	97.53	15.20	97.30	15.43

Results represent CD at 5% of three replicated experiments.
Treatments (A) = 0.17, N.S.; Cultivars (B) = 0.17, 0.44; A X B = 0.24, N.S.

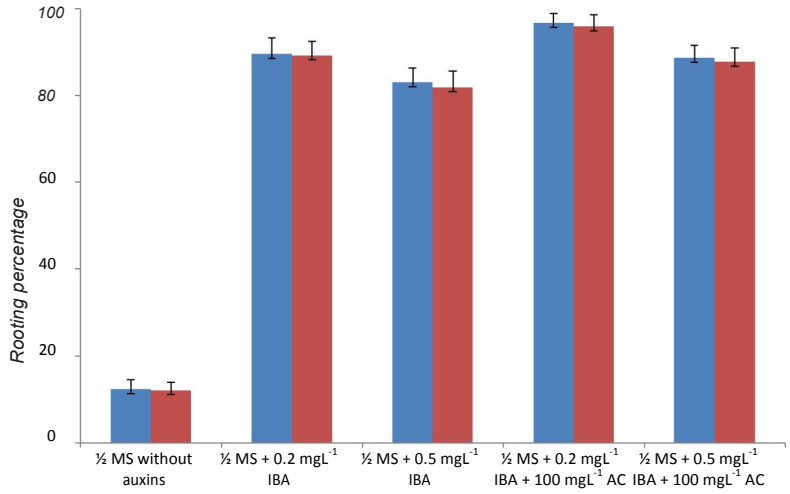

Fig.2: Effects of IBA and AC on rooting in two *stevia* genotypes CIM madhu and CIM mithi

DISCUSSION

Type of growth regulator and genotypes are considered to be important factors for callus induction. Several researchers reported the induction of callus from the leaf explants in *Anthurium andreanum* and *Stevia* (Atak & Celik, 2009; Patel & Shah, 2009).

Growth and developmental processes are generally regulated by growth regulators, which are present in various concentrations in different parts of plant and nutritive media. Phytohormones are essential to disturb the established polarity in the organ for the initiation of cell division. A requirement for exogenous auxin in callus initiation has also been established by Lahiri *et al.* (2012). The combination of a cytokinin with an auxin has been reported to strongly enhance callus induction in many plant species (George, 2008; Irvani *et al.,* 2010; Lahiri *et al.,* 2012). The combination of IBA and BAP may act synergetically with each other and induce the cells to dedifferentiate to form callus. In contrast, no callus induction from leaf

explants was seen with any concentrations of IBA in *Saccharum officinarum* (Gopitha *et al.,* 2010). Meanwhile, the frequency of callus induction may vary from one species to another due to the endogenous level of hormone, their uptake, type of auxins and cytokinins used and also on the mode of action (Gupta *et al.,* 2010).

Similar observation of emerging out green shoot buds from the surface was also observed by Irvani *et al.* (2010) in *Dorem ammoniacum*. Similar reports on efficacy of Kn over BAP are also available for other plant species (Kumar, 1992; Rahman *et al.,* 2004; Zibbu & Batra, 2010). The quantitative interactions between the appropriate plant growth regulators at optimum culture conditions play an important role towards the success of callus cultures (Benmoussa, 1996; Mukhopadhyay *et al.,* 2008; Atak & Celik, 2009). The *in vitro* response of plant tissues towards callus induction, growth and regeneration often seem to be under an over-riding genetic control with other factors exerting only a minor effect (George, 2008; Atak & Celik, 2009).

The roots emerged from the base of the regenerated shoots. The difference among plant parts to the IBA treatment may relate to the differences in their internal growth regulators. The efficiency of IBA in root induction from shoots *in vitro* has been reported for several plant species (Yadav & Singh, 2010; Singh *et al.,* 2010; Yadav & Singh, 2011). AC attributes to the reduction of light at the base of the shoots and provides the environment for auxins accumulation. Druart *et al*. (1982) found the positive response of AC on rooting.

The high survival rate of the regenerated plantlets under field conditions indicated their superiority with both physical and chemical environments.

CONCLUSION

In vitro methods provide an effective alternate means for rapid continuous multiplication of species to meet the demand for commercial exploitation. The genotype used and its interaction with the various physical and biological factors also influence the different stages of *in vitro* multiplication.

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