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Comparative studies of *in vitro* and *in vivo* grown plants and callus of *Stevia rebaudiana* (Bertoni)

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Abstract
Stevia rebaudiana (Bertoni) is investigated as an option for artificial sweetening agents. An efficient plant regeneration via shoot and callus organogenesis was established. Explants were cultured on MS medium containing different concentrations of cytokinins and auxins. Optimal shoot initiation was achieved on medium containing 0.3 mg/l kinetin. Root induction was optimized on MS medium with 2.0 mg/l IBA. Callus optimization was observed on MS medium supplemented with 0.1mg/l 2,4-D.Shoot initiation from callus was maximum in MS+BA +2,4-D and rooting was supported by presence of IBA in MS medium. Rooted plantlets were successfully acclimatized, with a survival rate of 40%.Plantlets and calli grown on MS medium with different plant growth regulators were screened for proximate analysis. Plant and callus regenerated from shoot explant on MS+2,4-D+IBA was proved to be a substantial source for stevioside production. In histological analysis vascular tissue development was seen.

Keywords: MS medium, callus, shoot generation, histological studies.

INTRODUCTION

Stevia rebaudiana (Bertoni) is a major source of high potency sweetener for the growing natural food market, in the future. Stevia is a perennial herb and belonging to the daisy family. It is a natural low caloric sweetner due to the stevioside contents in its leaves.

Stevioside is a glycoside and it has a sweetening power 100-400 times than sucrose (Gujaral 2004; Duke 1993). Uses of stevia are not only restricted as a sweetening agent but as a medicine and household purpose. It is recommended for diabetes and has been extensively tested on animals and humans without any side effects (Genus, 2003). Human body is not able to convert these glycosides in the body therefore have a glycemic index zero (Brandle and Rosa, 1992). Other attributes of this natural, high-intensity sweetener include non-fermentable, non-discoloring, heat stability at 1000C and feature a lengthy shelf life. The product can be added to tea and coffee or baked goods, processed foods and beverages (Gujaral, 2004).

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The propagation through seeds is not adequate owing to very low seed germination percentage. Vegetative propagation needs high labor input. Because of these problems large scale production has limitations. Demand for stevioside is high hence tissue culture is the only alternative for rapid propagation of stevia plants.

Plant regeneration from *in vitro* culture can be obtained by somatic embryogenesis or organogenesis. In *Stevia*, regeneration has been obtained by organogenesis from leaves (Ferreira and Handro, 1987; Ferreira and Handro, 1988; Yang and Chang, 1979), axillary shoots (Bespalhok *et al*., 1992), stem tips (Tamura *et al*., 1998), suspension cultures (Ferreira and Handro, 1988) and anthers (Flachsland *et al*., 1966). Although somatic embryogenesis has been reported previously from leaves (Bespalhok *et al*., 1993; Wada *et al.*, 1981) and stems (Miyagawa *et al.*, 1984) Tamura *et al.* (1998) established clonal propagation of *S. rebaudiana* by culturing stem tips with an increasing demand for stevioside in the food industries. Clonal propagation of *S. rebaudiana* has been established by culturing stem tips with a few leaf primodia on agar medium supplemented with a high concentration (10 mg/l) of kinetin. Ferreira and Handro (1988) described a method for production maintenance and plant regeneration from cell suspension cultures of *S. rebaudiana* (Bert.)

Bertoni. Bespalhok *et al.* (1993) established somatic embryogenesis from leaf explants of *S. rebaudiana.* Bespalhok and Hattor, (1997) obtained embryogenic callus formation and performed histological studies from *S. rebaudiana* (Bert.) Bertoni floret explants. Sivaram and Mukundan (2002) reported *in vitro* culture studies on *S. rebaudiana*.

Interest has been rekindled in more recent years, especially in the developed world where diet conscious consumers seek a natural low-calorie sweetener as an alternative to chemical sweeteners. The aim of present study was to observe effect of different growth hormones on optimization of tissue culture and stevioside production. Histological analysis was also performed to see effect of plant growth regulators on callus.

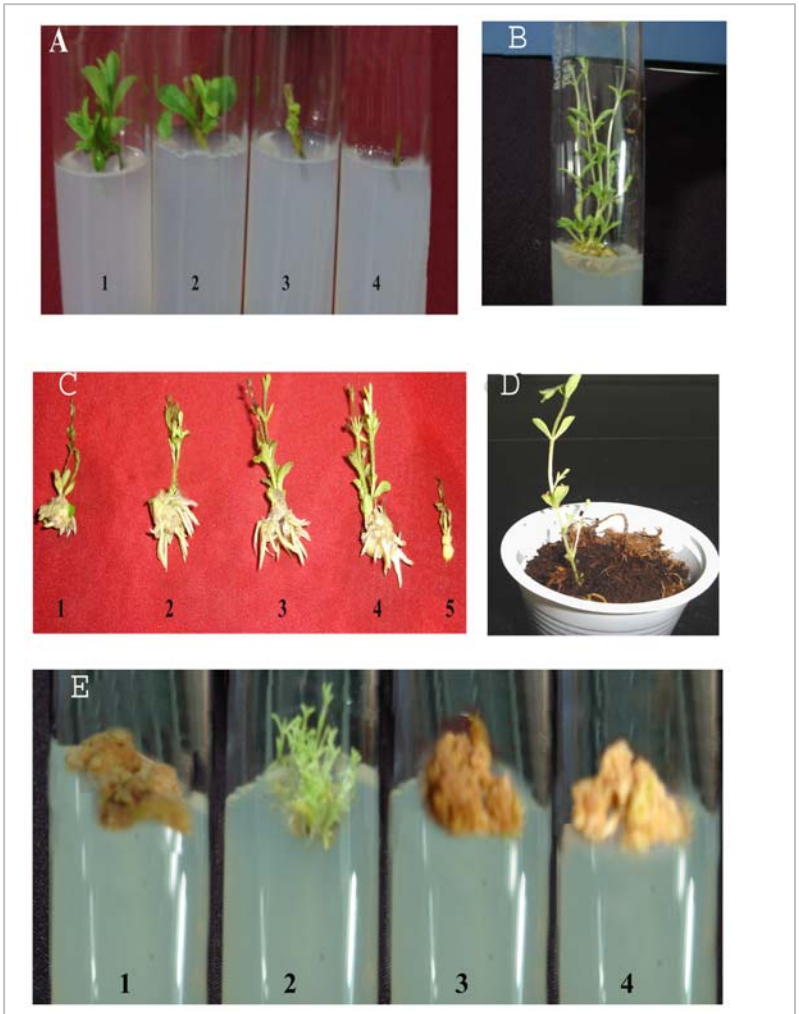

Figure 1: Effect Of Growth Hormones On *Stevia rebaudiana*
A. 1) MS+Kinetin 0.3 mg/l , 2) MS+BA 1.3 mg/l 3)MS+ Kinetin + BA 0.3 + 0.3 mg/l 4)Basal MS medium, **B.** Shoot Multiplication MS+ BA+ IAA (2.0+1.0 mg/l) , **C.** Rooting 1)MS+2,4-D (1.5 mg/l) 2) MS+IBA(1.5 mg/l) 3) MS+IBA (2.0 mg/l) 4) MS+ IBA +2,4-D (2.0 +1.5 mg/l) 5) MS+ IBA + IAA (2.0 +1.0 mg/l), **D.** Hardening of plantlet in sphagnum+ cocopeat (1:1) , **E.** Callus regeneration 1) MS+ BA+ IAA (1.5 +0.1 mg/l) 2) MS+ BA+ 2.4-D (1.5 +0.5 mg/l) 3) MS+ BA+ NAA (1.5 +0.5 mg/l) 4) MS+ BA+ IBA (1.5 +0.5 mg/l)

MATERIALS AND METHODS

Explant preparation

Internodes and apical shoot tip were used as explant for tissue culture experiment. The explants were collected from field grown plants at Ajeet Seeds Ltd, R&D Center, Aurangabad. Nodal stem segments and apical segment with 2 cm were excised from these plants, disinfected in a solution of sodium hypochlorite (0.4%) for 10 min followed by 0.1% $HgCl_2$ and then rinsed for three times with sterilized water.

Preparation of culture media and inoculation

Stock solutions of all the components of Murashige and Skoog (1962) medium were prepared with appropriate amount of all the components. Appropriate amount of stock solutions were mixed following the standard media preparation procedure. Full strength MS medium was used as a basal medium. It was supplemented with various growth plant growth regulators like kinetin (Ki), indol-3-Acetic acid (IAA), 4-indol-3-butyric acid (IBA), NAA, gibberelic acid (GA), 2,4-dichlorophenoxy acetic acid (2,4-D) in different concentrations and combinations. The pH of the medium was adjusted at 5.8 before autoclaving. All inoculations were carried out in a laminar air flow cabinet. Cultures then incubated in a culture room at 25^0C and a 16 hrs light-8 hrs dark period. Observations were taken at a regular interval for growth and contamination. If contamination was observed then cultures were removed from growth room.

For shoot regeneration from nodal segment Ms Medium was supplemented with Ki, BA alonely as well as in combination of two and auxins. Initiated shoots then transferred on multiplication medium. Multiplied shoots then used for rooting. For rooting MS medium was supplemented with auxins. Hardening of rooted plantlets was performed on a soilless medium.

Apical shoots were cultured on MS medium supplemented with auxins and combinations of various auxins for callus initiation. Callus multiplication was optimized on MS medium with growth plant growth regulators. Obtained callus then used for organogenesis.

Biochemical analysis

Calli obtained at various combinations of auxins, micropropagated plantlets, callus originated plantlets were used for various biochemical analysis. Calli used for analysis were obtained on MS+ 2,4-D+IBA, MS+2.4-D+GA, MS+2,4-D+ IAA, MS+2,4-D. Results were compared with commercial stevia powder. Stevia powder was purchased from Ayuer herb Mumbai.

In biochemical analysis estimation of carbohydrate was performed by phenol-sulphuric method (Sadasivam and Manickam,1992), proteins by Lowry method (Lowry *et al.*, 1951). Alkaloid was determined by (Harborne 1973), flavonoid by Boham and Kocipai-Abyazan (1974) terpenoids (Morigiwa 1986) steviosides (Nikolova Damyanova *et al.*, 1994). Estimation of phenols (Thimmaiah 1999) and tannin was performed by using Folin-Denis method (Sadasivam and Manickam 1992).

In further analysis total ash content was determined according to AOAC (1965). In the present experiment percentage moisture was calculated according AOAC (1984). Estimation of nitrogen was done by micro-kjeldal method (Sadasivam and Manickam 1992), Potassium by Jackson (1962), Amount of phosphorus was calculated using Fiske and Row method (1925). Estimation of calcium was performed according to Piper 1950.

Histological studies

For observing cell shape and arrangement in the callus, microtomy analysis was done (Mohanty 1998). First the samples were fixed in formalin-acetic acid-alcohol-water (50:5:10:35ml) mixture. After fixation samples were dehydrated using graded solutions of ethyl alcohol: butyl alcohol: distilled water. In these series samples were keep for one hr. in first four series and then 2 hours in rest. After dehydration samples were transferred to clearing solution. Samples were embedded in paraffin. Sections of 8µm were taken on a microtome. Sections were stained and observed under compound microscope.

RESULTS AND DISCUSSION

Micropropagation through nodal segments

In a significant development it was observed that shoot buds originated from nodal explants, when MS was supplemented with different concentrations (0.1-1.0 mg/l) of KI and (1.0- 1.5 mg/l) of BAP. The nodal explants showed emergence of shoot buds developing from the pre-existing material seven days after inoculation.

Shoot initiation was also seen on MS fortified with kinetin and BA along with combination of auxins. But a very low length of shoots was developed in the combination of kinetin and auxins. Optimum shoot length was seen in the MS medium supported with 0.3 mg/l. BA at 1.3 mg/l and combination with auxins were also proved somewhat supportive for shoot initiation (Table 1 *[Supplementary data]*, Fig. 1A). Similar studies on shoot proliferation has been performed by Patil (1996), Nepovin and Vanek (1998), Sikach (1998), Akita and Shigeoka (1994), Ferreira and Handro (1998) and Sivaram and Mukundan (2003). They also reported that plant hormone is necessary for shooting, elongation and rooting. In most of cases BAP was found to be essential for growth and was better than Ki for shoot induction for various explants. Combination of BAP and IAA has also been reported to be better for shooting and elongation. Tamura *et al.*, (1984) in another work established stevia propagation with leaf primordial with 10 mg/l kinetin in a MS medium. Kinetin concentration searched out in the present study is quite low with respect to other studies. It may be due to the endogenous level of growth plant growth regulators in the experimental plant.

After shoot initiation, various combinations of cytokinins and auxins were checked for shoot multiplication. In these treatments, combination of BA and IAA showed excellent length of shoots (2.78 cm) and shoot multiplication rate of five folds over the other treatments (Table 2 *[Supplementary data]*, Fig. 1B). As multiplication cycle is of short duration repeated multiplication is possible. Thus the process provided a consistent and abundant source for highly regenerative material. This is in accordance with Sivaram and Mukundan (2002) who established clonal propagation of *Stevia* by using nodal segment.

Multiplied shoots further used for root initiation. Different combinations of auxins were tried with MS medium for root initiation. 100% rooting percentage and optimum root length was found in the treatment of 2.0 mg/l. IBA (Table 3 *[Supplementary data]*, Fig. 1C). Other treatments were not able to induce proper roots. Rooting with IBA was earlier reported by Pande *et al*., (2002) in *Ammi majus*, Raha and Roy (2001) in *Holorrhena antidysentrica*, Saini and Jaiwal (2000) in *Peganum harmala*, Hussain *et al*., (2007) in *Sterculia urens*, Joshi and Dhar in S*aussurea obvallata*.

Micropropagation through callus

Out of various concentrations of 2,4-D used optimum biomass was observed in the concentration of 0.1 mg/l. In the treatment of 2,4-D and IAA increase in callus biomass was superior but showed callus browning at higher concentrations. Same results were also obtained with IBA and 2, 4-D. With 0.3 mg/l concentration of IBA gave comparatively good callus growth.

Cytokinins and gibberllins with 2,4-D were tried for obtaining callus multiplication. Combination of 2,4-D and cytokinins were not showing callus multiplication. Satisfactory results were observed in the treatment of GA (0.1 mg/l) (Table 4 *[Supplementary data]*, Fig. 2a *[Supplementary data]*) Callus formation has also been observed by Xie *et al.*, (1998), Bespalhok and Hatori (1997), Ouyang (1996), Ferreira and Handro (1988) from leaf and floret (Flachsland *et al.*, 1966) of *S. rebaudiadia*.

Shoot initiation from callus was observed in the combination of BA and 2,4-D (1.5+0.5 mg/l) (Fig. 1E). The results are supportive to the work carried out by Bhojwani and Rajdan, 1996. Variations in the results may be due to the endogenous phytohormone contents, their uptake, the type of auxin and cytokinin used and their mode of action (Tran Thanh Van and Trinh, 1990).

Biochemical analysis

In rooting of shoots as compare to the 2,4-D treatment, IBA induced high number of roots per shoot and root length was high. In the various concentrations of IBA 1.5 mg/l showed acceptable root initiation. Other hormonal combinations were not giving expected results. Debnath (2008) also reported the same treatment for *Stevia*. Further results are in accordance with *Carica papaya* in which plantlets were rooted in full strength MS medium with IBA (Yang and Ye 1992).

Obtained were hardened on cocopeat and moss. After twenty-five days, survival percentage was evaluated. Survival percentage was found more 40% in the proportion of 1:1 (Fig. 1D).Good harden plants then transferred to glass house. Successful acclimatization of microshoots was done on the mixture of cocopeat and moss. After primary hardening of twenty –five days plantlets were transferred to glasshouse with 40% survival. Successful hardening with peat and moss also reported for *Lilium speciosum* (Chang *et al.*, 2000), *Adenophora triphylla* (Chen *et al.*, 2001).Morphological variations have not been observed in the regenerated plants. This indicates that phytohormones used in present investigation were suitable for clonal propagation of *Stevia.*

In the third part of the work various biochemical analysis were done. For biochemical analysis calli obtained from MS+ 2,4-D+IBA, MS+2.4-D+GA, MS+2,4-D+ IAA, MS+2,4-D, plantlets from shoot proliferation and plantlets obtained from callus organogenesis were used. This is the first type of work where comparative studies are performed to know difference and similarities within *Stevia*. Results are compared with the commercial S*tevia* powder.

The proximate analysis showed that all the calli and plantlets are good source of carbohydrates except callus developed on combination of 2,4-D and GA. Plantlet obtained through exhibited highest carbohydrate percentage (51%) even more than commercial stevia powder(40%). Range of carbohydrate content is additional according to Tadhani and Subhash (2006a) who carried out analysis of stevia and reported carbohydrate percentage 35.20. In another study carried out by Savita *et al.*, (2004) carbohydrate percentage in stevia is 52. This variation in carbohydrate content may be due to the variation in *Stevia* plant. Variations observed in the present investigation are may be because of hormonal effect on cultures. Same result trend was followed for protein where lowest protein content was observed in callus multiplied on 2,4-D with GA and 2,4-D with IBA. Literature states that protein content in *Stevia* is 20.42% according to Tadhani and Subhash (2006a) and 10% in accordance with Savita *et al.*, 2004.

In present protein estimation, highest content was found with shoot proliferative plant which is 26%. In both the cases callus multiplied on 2,4-D with GA showed lowest carbohydrate and protein content that is may be due to effect of GA which said to be inhibitory on carbohydrate production Literature supports to the results as similar observation reported in *Tulip* where carbohydrate content was decreased after application of GA in the field (Geng *et al.*, 2007). Same observations were recorded in Strawberry (Mohamad *et al.*, 1991). But opposite development was reported in Maize, cowpea, broad bean in which carbohydrate increased after application of GA (Ahmed *et al.*, 1989).

The presence of secondary products in the samples that are biologically important e.g. alkaloids, flavonoids, terpenoids, glycosides, tannins, phenols contribute to its medicinal value as well as exhibiting physiological activity (Sofowara 1993). Plantlets obtained through shoot proliferation and callus organogenesis were proved to be substantial source for alkaloids. But alkaloid contents were highest in commercial powder and lowest in the callus grown on 2,4-D+GA. In the previous studies quantity of alkaloids was not recorded only presence was reported by Tadhani and Subhash (2006a). Flavonoids were only present in the plantlets and quantity was lower than commercial stevia powder. Again terpenoid quantity was high in plantlets only. These variations are may be because of inappropriate combination of phytohormones for alkaloids, flavonoids, terpenoids.

Stevioside quantity was found to be similar in the shoot originated plantlet which was one month old in green house and commercial powder which was extracted from fully matured plant. Various calli also showed much higher level of stevioside. Among different calli , the callus developed on combination of 2,4-D and IBA was proved to be substantial source of stevioside

According to previous study carried out by Swanson *et al.* (1992) callus was not able to synthesize stevioside but in the present experiment calli also proved to best for stevioside production. In an another recent comparison of stevioside content from *in vitro* and *ex-vitro Stevia rebaudiana* highest stevioside contents were recorded in micropropagated plants (Fig. 3) (Rajasekaran 2006).

Presence of phenol and tannin was recorded in all samples. Total phenol and tannin contents were low as compare to commercial stevia powder. In the earlier research by Tadhani and Subhash (2006a), Savita *et al.*, (2004) phenol and tannins were reported in Stevia. Tannins have been reported to have several pharmacological activities in smooth muscle cells (Tona *et al.*, 1999).

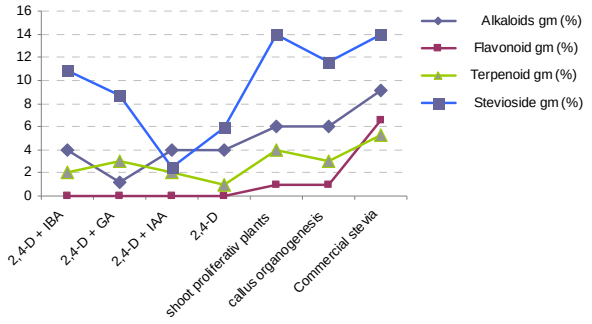

Figure 3: Secondary metabolite contents in different calli and plantlets

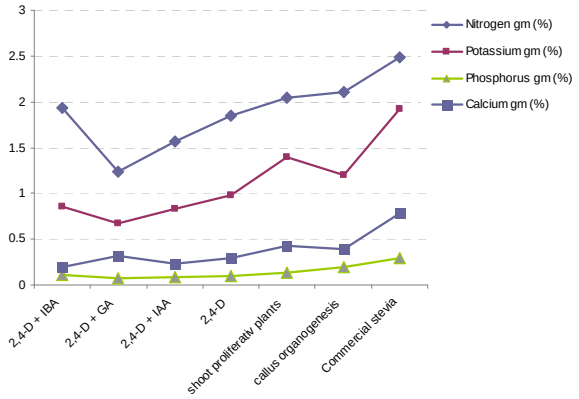

Figure 4: Nitorgen, potassium, phosphorus and calcium contents from different calli and plantlets.

Callus grown on MS+ 2,4-D+GA and MS+2,4-D showed highest ash content i.e. 8.96% and 8.11% respectively. High ash content indicates that the calli are good source of inorganic material (Tadhani and Subhash 2006).

Nitrogen, potassium, phosphorus and calicium which are nutritionally important were found in a reasonable amount in samples. Highest content were estimated in the fully grown plantlets as compared to the calli (Fig. 4). The difference is may be because of minimum synthesis in calli. In the previous studies completed by Tadhani and Subhash (2006a) and Savita *et al.*, (2004) recorded presence of minerals.

Histological analysis

In histological studies callus showed parenchymatous cells. GA showed thick walled parenchymatous cells while in other thin walled parenchymatous cells were observed. IBA was responsible for differentiation of vascular tissue (Fig. 2b) Results are in accordance with *Phoenix dactylifera* (Sane, *et al.*, 2006) *Allium sativum* (Fereol *et al.*, 2005), *Kigelia pinnata* **(**Thomas and Puthur 2004), *Bauhinia Forficate* (Mello *et al.*, 2000).

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