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IN-VITRO PROPAGATION OF SWEETENING CROP PLANT STEVIA RABUADIANA THROUGH APICAL AND AUXILIARY NODS

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ABSTRACT

Stevia rebaudiana has an estimated 300 times sweeter than the cane-sugar. The sweet taste of the vegetative part of the plant is due to the presence of diterpene glycosides, viz. stevioside and rebudioside. Apart from its huge commercial demand as sweetener in food industry, it has also got several medicinal values. *Stevia rebudiana* is being used in herbal therapy by the native peoples of Brazil and Paraguay for long time. Stevia produces few seeds with very low germination percentage. Keeping in view increasing demand of stevia it is important to deduce methods for large scale plantlets production. The micro propagation of epical and auxiliary nod to obtain sufficient amount of uniform and disease-free plants has been studied in present investigation. The effects of different tissue culture basal media containing IAA, IBA and Kinetin were studies. The obtained micro-shoot was found suitable both for further multiplication and or transferring to the pots or field in greenhouse conditions.

Key word: *Stevia rebaudiana*, auxillary bud, epical bud, micropropagation, sweetner

INTRODUCTION

Stevia rabuadiana belongs to the family Asteraceae. It is perineal herbaceous wild plant native to certain regions of South America like Brazil and Paraguay (Katayama *et al*., 1976). Moises Santiago Bertoni (1905) was the first to describe the sweet taste of *Stevia rabuadiana*, then in 1931 Bridel & Lavielle reported the active components Glycosides, Stevioside and Rebaudioside that gives the sweet taste to Stevia. Stevia had

been used for removing the bitter taste of herbal medicines. These compounds are 300 times sweeter than sugar and cannot be analyzed or absorbed by human digestive system. Consequently stevia does not have any effects on blood sugar (Taware *et al.*, 2010). The native people of Paraguay use it as digestive and superficial ointment (Elkins, 1997). It stimulates the secretion of insulin from pancreas due to its antihyperglycemic nature (Lu, 1993; Tomita, 1997). Stevia is therefore an attractive natural sweetener to diabetic patients and others who are conscious about carbohydrate controlled diets (Gegerson *et al.*, 1972). Stevioside is not only diuretic but it also possesses the antibacterial properties and dispensed in treating wounds, sores and gum diseases. Stevia contribute 40% of market sweetener in the world. It is used as sweetener in food products and soft drinks in Japan.

For commercial scale cultivation of *S. rebaudiana*, its poor seed germination is a limiting factor. However, its propagation is usually done by stem cuttings, which root very easily but requires high labour that make it a costly. The resulted plants through stem cutting show great variety in important characteristics like sweetening level. In addition, propagation through stem-cutting is limited by stocks of stevia stem and enough time for obtaining them from single plants (Sivaram *et al.,* 2003). Furthermore, due to the low primary growth, the seedling is not able to compete with weeds (Jain *et al*., 2009). In such conditions, the micro propagation through tissue culture is the only method to support large scale propagation in rapid, less laborious and cost effective mean.

Protocols for propagation of *S. rebaudiana* from, root, leaf, nodal and axillary shoot explants are established in different countries (Yang *et al*., 1981; Lu, 1993, Ghauri *et al*., 2013). In the present studies the micro propagation of *Stevia rabaudiana* apical and axillary nodes explants has been established in India.

MATERIALS AND METHODS

Explants collection:

The shoots of actively growing *S. rebaudiana* were collected as source of explants from the research field of Center. Segments of apical and axillary nodes about 1.0 cm in length were isolated from the shoots. The explants were washed in running tap water and then washed again roughly by adding a few drops Tween-20 to remove superficial dust and infections. Finally the explants were surface sterilized with mercuric chloride ($HgCl_2$) solution under aseptic condition and then washed three times with sterilized distilled water. Root explants were cultured on

Murashige and Skoogs medium (Murashige & Skoog, 1962) supplemented with 2% (w/v) sucrose.

Culture Conditions and Growth media: The MS medium along with 100 mg/l myo-inositol, 30 g/l sucrose, 0.4 mg/l thiamine HCL and 7 g/l agar was used as basal nutrient medium. The pH of 5.7 to 5.8 was adjusted using few drops of either 0.1 N NaOH or 0.1 N HCl. The media was distributed in screw capped culture vessels. Sterilization of the medium was achieved by autoclaving the tubes containing media under pressure of 15 psi for 20 minutes. One explants was placed in each test tube cultures that were incubated in a growth chamber at 25°C for 16-h/day photoperiod using cool white fluorescent lamps (300 Lux). The explants were maintained by regular subcultures at 4-week intervals on fresh medium with the same composition.

Culture Establishment: MS medium were supplemented with 6-benzylamionpurine (BAP) at the 0.0, 0.1 and 0.5 mg/L concentration were inoculated with the explants alone or in combination with 0.0, 0.1 and 0.5 mg/l concentration of kinetin (kin-). The observation was recorded as Survival percentage, growth percentage to survival and average shoot length after 6 weeks from culture on establishment medium.

Shoot Multiplication: The most vigorous shoots from the establishment stage used for the multiplication experiments. 2-3 cm long explants with two axillary buds were cut and cultured in MS medium containing the same composition of BAP and Kin used in the establishment. Shoot proliferation was recorded after six weeks as number of new shoots formed per explants and average shoot length (cm).

Root induction and Acclimatization: Individual shoots of 3-5 cm long were excised from the multiplication media and transferred to MS basal medium containing the previously mentioned composition and supplemented with activated charcoal the Naphthalene acetic acid (NAA), each at 0.5, 1 and 2 mg/l concentrations. The percentage of rooted shoots, number of roots formed per shoot and average root length (cm) were determined after 6 weeks of culture on the rooting medium.

Rooted micro shoots were removed from the medium and the roots were washed thoroughly in sterile distilled water. These plantlets were transferred to pots containing peat moss and sand (1:1) in the greenhouse at 28 ± 2°C, RH 70-80% conditions. The potted plants were covered with plastic bags

initially which were gradually eliminated within four weeks time for completing their acclimatization. Irrigation was done regularly during acclimatization.

RESULT AND DISCUSSION

Among the explants used for the culture inter-nodal segments were found to be able to produce profuse shoots the results of induction and multiplication are presented in Table **1**. The result of the experiment and other earlier research report clearly support the possibility of propagating *S. rebaudiana* by adopting in vitro techniques. The climatic requirements of this tropical elite medicinal plant indicate that it can be introduced in the M.P. and Chhattisgarh region of India. The unique selling points of Stevia sweetener are very strong in India due to the presence of diabetic and other metabolic disease including obesity. Here Stevia *in-vitro* propagation has been demonstrated with its overall potentiality and suitability. In vitro propagation can important alternative to conventional propagation and breeding procedures for wide range of plant species. The explants (shoot meristem) shows batter response when collected and inoculated form August to march. The explants sterilization for 8-10 minutes with 0.1% $HgCl_2$ solution shows 70-80% sterile culture.

Establishment: In the preliminary experiment effect of cytokinins alone or in combination were tested. Maximum (70-80 %) number of bud break and initiation of shoot was reported in BAP alone (0.5-1.0mg). About 1-2 shoots were developed. The combination of BAP and Kinetin contained media shows 40-50% of initiation of shoot was found. Maximum length of shoots was observed 1-2 cm in the medium containing BAP alone in comparison to medium supplemented with BAP and Kinetin **(Table 1)**. Higher concentration of BAP gives short and week shoots with the formation of non fragile callus **(Fig. 1)**. Similar results were reported by Sane *et al* (2001) who reported the stimulation of shoot in case of accasia. The result is also similar to the one of Skolmen and Mapes (1976) and Dhawan and Bhojwani (1985) in case of Accasia and Leucaephala respectively.

Shoot multiplication: In order to optimize a suitable medium for mass multiplication of shoots from a single initiated nodal region, the highest number of shoots was observed in the medium containing higher concentration of BAP (1.0-2.0 mg/1) shown on **Table 2** and **Fig. 2**. These media show about 15-20 number of shoots per culture, when subcultured in the same fresh medium after 15 days duration. The length of shoots

elongated 4-5 cm and increases with culture duration. About 5-9 shoots with 2-3 cm were achieved in the medium containing Kinetin and BAP, and produces callus. For further improvement in proliferation shoots were subcultured in the medium containing same BAP concentration. Cultures shows better response in shoot number (20-25) and shoot length (3-5cm) in the medium. Thus the medium containing BAP (1.0-2.0 mg/1) were standardized as the best media for mass production and elongation of *Stevia rebaudiana*. The result observed is supported by the reports by Ghauri *et al* (2013) for micro propagation of Stevia rebaudiana in Pakistan. Komalavalli (2000) and Raha and Roy (2001) for *Holarrhena antidisenteria* reported similar results.

Rooting: The multiplied shoots of 2-3 cm shows 80-90% rooting in the medium containing Activated Charcoal and NAA, About 2-4 thin long roots were developed which increases with the age of culture. Root length recorded was 2-8 cm, in the same medium **(Table 3)**. The medium supplemented with reduced sucrose does not shows very good response may be due to low sugar and without growth regulators while satisfactory response on rooting has achieved in the medium containing NAA **(Fig. 3.)**. This results is supported by the observation made by Abd Alhady *et al*, (2010) whereas contradicts the findings of Mohammad and Alhadi (2011).

Table 1: Effect of growth regulations on shoots induction in *Stevia rebaudiana*

S No	Medium Growth hormones mg/l	% age of shoot induction	No of shoots per culture	Average shoot length in cm	Callusing
1	MS+ 0.5 BAP	70-72%	1-2	1-2	-
2	MS+ 1.0 BAP	75-78%	1-3	2	-
3	MS+ 1.5 BAP	67-70%	1-2	2	-
4	MS+ 2.0 BAP	60-65%	1-2	1-2	+
5	MS+0.5 KN	40-46%	1	1-2	++
6	MS+1.0 KN	42-48%	1	1-2	
7	MS+0.5 BAP +0.5 KN	45-47%	1	1	++
8	MS+1.0 BAP +0.5 KN	38-45%	1-2	1	+

Table 2: Effect of growth regulations on multiplication of shoots in *Stevia rebaudiana*

S No	Medium Growth hormones mg/l	% age of shoot response	Average no of shoots	Average shoot length in cm	Callusing
1	MS+ 1.0 BAP	70	15	2-3	-
2	MS+ 2.0 BAP	80	15-20	2-3	-
3	MS+ 2.0 BAP P+0.5 KN	60-65%	8-10	2-5	+
4	MS+ 3.0 BAP	50	4-5	3-5	-
5	MS+1.0 BAP+1.0 KN	50	5-8	1-2	+
6	MS+1.0 BAP +2.0KN	43-45%	4-9	1-2	++

Table 3: Effect of different media on root induction in *stevia rebaudiana*

S No	Medium	% age of shoot rooting	Root length (cm)	Root Morphology
1	MS+ 10g/1sucrose	58-60	2-3	Thin, Short
2	MS+ 1.0mg/1 NAA+20g Sucrose	80-85	2-5	Thin, Short
3	MS+ 200mg activated Charcol	85-90	3-5	Thin- Long

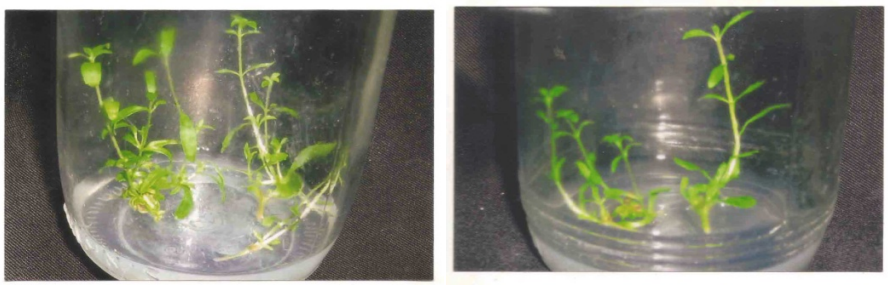

Fig. 1 Induction of shoots from explants

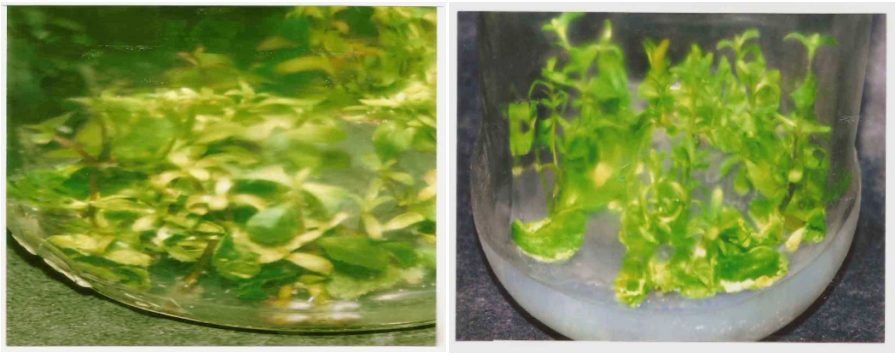

Fig. 2: Shoot multiplication

Fig. 3: Elongated rooted shoots.

CONCLUSION

The in-vitro micropropagation of *Stevia rebaudiana* is successfully established. The observation suggests that the combination of BAP and kinetin could be used for shoot multiplication as well as establishment. The root induction is positively influenced by the presence of growth regulator NAA and activated charcoal in the rooting medium.

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