In vitro Micropropagation of Stevia rebaudiana (Bert.)

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Abstract: Stevia rebaudiana Bert., one of the important medicinal plants was subjected to in vitro multiplication. Present study shows a brief review of micropropagation of Stevia rebaudiana by using various basal salts that is Murashige and Skoog (1962) (MS), ½ Murashige and Skoog (MS) medium, Schenk and Hildebrantdt (S & H) medium supplemented with different concentration of BAP at (0.0, 0.5 and1.0 mg/l) or concentration of NAA at (0.0, 0.5 and 1.0 mg/l) was investigated on in vitro shoot multiplication of Stevia rebaudiana (Bert.) using shoot tip explants. The best results were obtained from S & H medium supplemented with BAP concentration of 1.0 mg/l respectively for shoot multiplication. For other characters viz; Average number of shoots and length of shoot, S & H was better than other basal salt and gave highest callus growth.

Keywords: Stevia rebaudiana (Bert.), MS medium, ½ MS medium, S & H medium, in vitro shoot multiplication, Multiplication Rate.

Introduction

Stevia rebaudiana (Bert.) is an herbaceous perennial plant of the Asteraceae family [(30)], native to Paraguay (South America). It is a natural non caloric sweet testing plant used around the world for its intense sweet taste. Diterpene glycosides produced by *Stevia rebaudiana* (Bert.) leaves are many times sweeter than sucrose. They can be utilized as a substitute to sucrose [(29,30,31, 15,16,17)]. The seeds of *Stevia* show a very low germination percentage. Propagation by seeds does not allow the production of homogeneous populations, resulting in great variability in important features like sweetening levels and composition [(22)]. Vegetative propagation too is limited by the lower number of individuals that can be obtained simultaneously from a single plant [(34)]. Due to the above-mentioned difficulties, tissue culture is the only alternative for rapid multiplication of *Stevia rebaudiana* (Bert.), plants.

The present study was aimed to understand the effect of different basal salts and different plant growth regulators at various concentrations on in vitro shoot induction and indirect plant regeneration of *Stevia rebaudiana* (Brert.). During process of micropropagation all types visible infections are rejected to maintain aseptic culture condition. The resultant micropropagated plants are disease free and healthy.

Materials and Methods

Plant Collection: 1cm length of shoot tip segments of *Stevia rebaudiana* (Bert.) collected from the established *in vitro* shoot cultures at Tissue culture Labortory, Cadilla Pharmaceutical limited, Ahmedabad, were used for experiments.

Culture Medium: Separate stock solutions were prepared according to the composition of the medium and were stored at 7-8 °C temp. The pH of the media was adjusted to 5.8 ± 0.1 using 0.1 N NaOH or 0.1 N HCL. Various concentration of Cytokinin (BAP) in combinations with Auxin (NAA) and alone i.e. T_1 (BAP 0.0 mg/l, NAA 0.0 mg/l), T_2 (BAP 0.05 mg/l, NAA 0.0 mg/l), T_3 (BAP 0.10 mg/l, NAA 0.0 mg/l), T_4 (BAP 0.0 mg/l, NAA 0.05 mg/l), T_5 (BAP 0.05 mg/l, NAA 0.05 mg/l), T_6 (BAP 0.10 mg/l, NAA 0.05 mg/l), T_7 (BAP 0.0 mg/l, NAA 0.10 mg/l), T_8 (BAP 0.05 mg/l, NAA 0.10 mg/l), T_9 (BAP 0.10 mg/l, NAA 0.10 mg/l)were incorporated in MS, ½ MS, S & H medium along with 3 % sucrose and 0.7 % agar and then observations were recorded.

Culture Condition:

All the cultures were incubated under in growth room with 16 hr photoperiod of light having 65.33 µ $molm^{-2}$. S^{-2} intensity (1500 flux). Temperature of growth room was maintained at 26 ± 2 °C with 55-60 % relative humidity.

Results and Discussion: -

In the present experiment, attempts were made to study the effect of various basal salts and phytohormones on various aspects of shoot multiplication in *Stevia rebaudiana* (Bert.). Various concentration and combination of BAP and NAA were used in MS medium, ½ MS medium, and S & H medium medium, and observed for the morphogenic responses of explants of *Stevia rebaudiana* (Bert.).

The results of each of these aspects are presented and discussed here with considering observations and pooled observations for different characters under study.

Effect of Basal Media and Phytohormones on growth of explants (MS, ½ MS, S & H)

Average No. of shoot:

After 30 days of inoculation, the highest number of shoot induction was observed in Treatment T_5 (5.83) and the lowest average number of shoot was found in Treatment T_4 (0.48) in MS basal media. In the ½ MS media the highest average no. of shoot induction was observed in Treatment T_9 (5.61) and the lowest average no. of shoot induction was found in Treatment T_4 (0.93). In the S & H media the highest average no. of shoot induction was observed in Treatment T_9 (8.66) and the lowest average no. of shoot induction was found in Treatment T_7 (1.28).

This result indicates that the high level of Cytokinin alone gives best number of shoots as compared to combination with Auxin and absence of Cytokinin in medium. Same result shows that high level of Cytokinin proved best for the shoot induction as compared to combination with Auxin in this plant which is supported by[15]. He reported that the highest Numbers of Shoots produced on medium supplemented with BAP alone were best then other media.

Average Length of Shoot:

After 30 days of inoculation, the highest length of longest shoot was measured in Treatment T_6 (2.52 cm) and the lowest length of shoot was measured in Treatment T_4 (0.77) in MS basal media. In the ½ MS media the highest length of longest shoot was measured in Treatment T_3 (1.55 cm) and the lowest length of shoot was measured in Treatment T_4 (0.84 cm). In the S & H media the highest length of longest shoot was measured in Treatment T_4 (4.08 cm) and the lowest length of shoot was measured in Treatment T_9 (2.47 cm). same results show that the high level of cytokinine proved best for the length of shoot reported by [(15)].

Average Number of Internodes:

The data on average no. of internodes collected after 30 days revealed that the highest no. of internodes were observed in Treatment T_5 (2.28) and the lowest no. of internodes was obtained in Treatment T_4 (0.60). In the ½ MS media the highest no. of internodes was observed in Treatment T_6 (1.77) and the lowest no. of internodes was obtained in Treatment T_1 (1.21). In the S & H media the highest no. of internodes was observed in Treatment T_4 (3.61) and the lowest no. of internodes was obtained in Treatment T_1 (2.77). High level of Cytokinin and lower level of NAA combination is proved best for more growth of internodes as compared to Auxin alone. Similar results were reported by [(15)].

Root induction:

After 30 days of culturing, no root induction was observed in any Treatments in MS and ½ MS media. In S & H medium, the root induction was observed in Treatments T_1, T_4 and T_7. There was no root induction in other treatment as data indicated.

Multiplication Rate:

After 30 days of culturing, In MS medium the highest multiplication rate was observed in Treatment T6 (3.17), whereas the lowest multiplication rate was observed in Treatment T7 (0.32). In ½ MS medium the highest multiplication rate was observed in Treatment T_6 (2.43), whereas the lowest multiplication rate was observed in Treatment T_4 (0.60). and in the S & H medium the highest multiplication rate was observed in Treatment T_5 (5.28), whereas the lowest multiplication rate was observed in Treatment T_1 (1.66). Similar results were reported by [(36).] For no. of shoots, S & H is the best which indicated the more requirement of NH_4^+ for shoot development of *Stevia rebaudiana* (Bert.). By exhibiting the normal behaviour of cytokinin i.e. BA at 1.o mg/l, S & H basal gave the highest multiplying rate among all treatment under study. It also supports the requirement of NO_3^- over NH_4^+. The poorest M.R. were recorded in ½ MS salts indicated the imbalance of NH_4^+/ NO_3^- which was not suitable for *Stevia rebaudiana* (Bert.). The normal effect of cytokinin and auxin was not seen in MS Medium also. The multiplication rate was also lower then S & H salts. It was may be due to lower NH_4^+ and higher NO_3^-.

For the study it can be concluded that *Stevia rebaudiana* (Bert.) requires acidic phase of medium. High ratio of $NH4^+$/ NO_3^- and presence of higher Ca^{+2} and SO_4^{-2} were also beneficial for the higher multiplication. *Stevia rebaudiana* (Bert.) also prefers the low salt combination for the best results.

Conclusion:

In present experiment, single shoot of *Stevia rebaudiana* (Bert.) was used as an explants for different experiment based on the purpose of study, different level of phytohormones, basal salts and their combination were made to study their response on explants growth and development. Main findings of study for the characters viz, average no. of shoot, average length of shoot, average no. of internodes, multiplication rate, root induction, and callus growth are concluded as follows.

Role of Cytokinin and Auxin in shoot multiplication during in vitro culture was proved by many workers[14,15,16,17]. In case of *Stevia rebaudiana* (Bert.), present study proved that ratio of NAA 1.1 and BAP 1:2 gave best growth in S & H media in number of shoots, length of shoot, number of internodes, multiplication rate as compare to full MS, and ½ MS medium. More no. of shoot in the media supplemented with BAP in fast growing, may gave more no. of shoot per explants. It indicated that the S & H was the best performer over all the comparison with MS, and ½ MS medium. The combination of BAP and NAA found the best for increasing the length of shoot and over all S & H was the best performer compare with other basal media. Only BAP levels found the best for increasing multiplication rate. From the data it can be concluded that S & H favours the callus induction in *Stevia rebaudiana* (Bert). Further studies to identify the effect of basal salts on root induction are proposed for fine-tuned commercial protocol for large scale multiplication of *Stevia rebaudiana* (Bert.).

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Table – 1: Detailed Composition of the media is given in table (a) Composition of plant tissue culture media (Murashige and Skoog (1962) (MS), ½ Murashige and Skoog (MS) media, Schenk and Hildebrandt (S & H) media.

CONSTITUENT	**MEDIA (mg/l)**		
Macro Elements	**Full MS**	**½ MS**	**S & H**
Ammonium nitrate	1650	825	-
Potassium nitrate	1900	950	2500
Calcium Chloride.2H2O	440	220	151.02
Magnesium Sulphat.7H2O	370	185	195.34
Potassium dihydrogen Phosphate	170	85	-
Calcium nitrate	-	-	-
Potassium Sulphate	-	-	-
Ammonium Phosphate	-	-	300
Micro Elements			
Boric acid	6.2	6.2	5.00
Potassium Iodide	0.83	0.83	1.00
Sodium Molybdate.2H2O	0.25	0.25	-
Cobalt Chloride.2H2O	0.025	0.025	-
Cobalt Chloride.6H2O	-	-	0.10
Manganese Sulphate.2H2O	22.3	22.3	-
Manganese Sulphate.H2O	-	-	10.00
Zinc Sulphate.7H2O	8.6	8.6	-
Copper Sulphate.5H2O	0.025	0.025	0.20
Ferric Sulphate.7H2O	27.8	27.8	15.00
Na2EDTA	37.3	37.3	20.00
Molybdic acid	-	-	0.10
Vitamins			
Nicotinic acid	0.5	0.5	5.0
Pyridoxine hydrochloric acid	0.5	0.5	0.5
Thiamine hydrochloric acid	0.5	0.5	5.0
Myoinositol	100gm	100gm	100gm
Glycine	-	-	-
Sucrose	30gm	30gm	30gm

Table – 2: Different Combination of Phytohormones and Basal medium.

Auxin (mg/l)	**Cytokinen (mg/l)**			
	BAP / NAA	0.0 (mg/l)	0.5 (mg/l)	1.0 (mg/l)
	0.0 (mg/l)	T_1	T_2	T_3
	0.05 (mg/l)	T_4	T_5	T_6
	0.10 (mg/l)	T_7	T_8	T_9

Figure 1: - *In Vitro* Growth in full MS Media Supplemented with Cytokinin and Auxin.

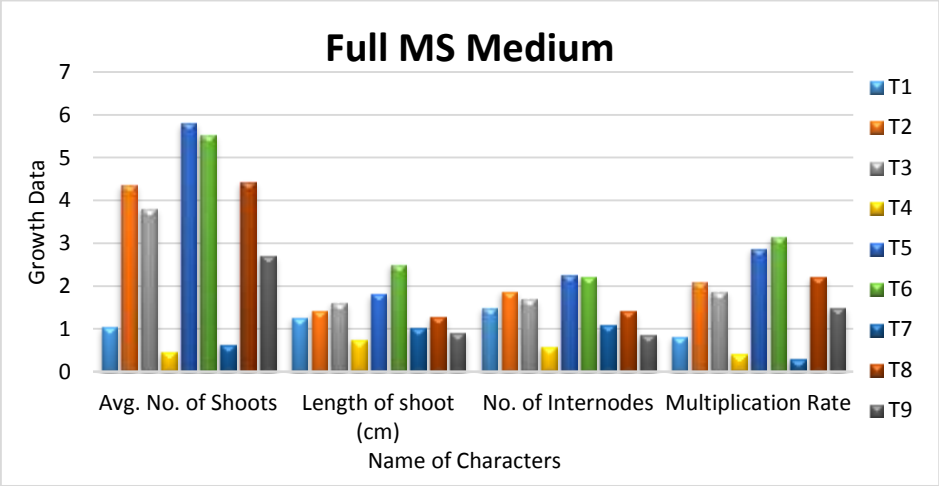


Figure 2: - *In Vitro* Growth in ½ MS Media Supplemented with Cytokinin and Auxin.

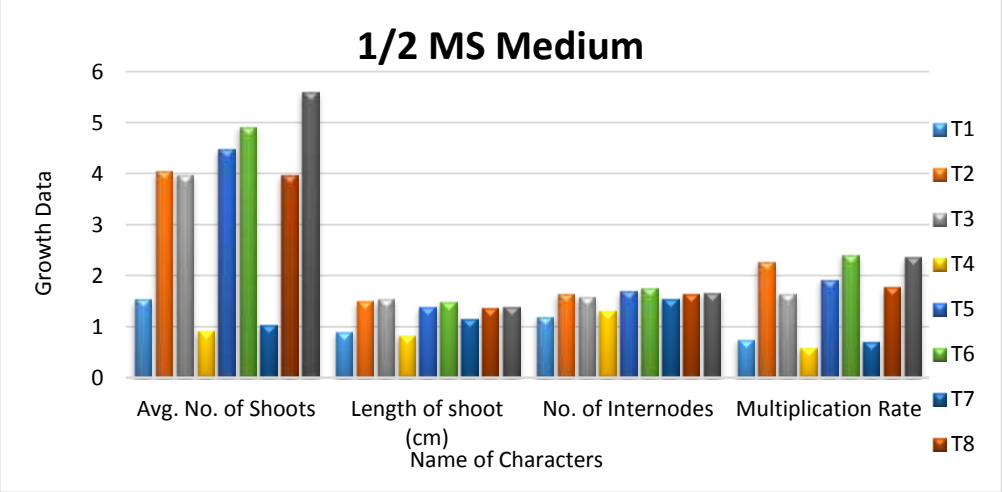
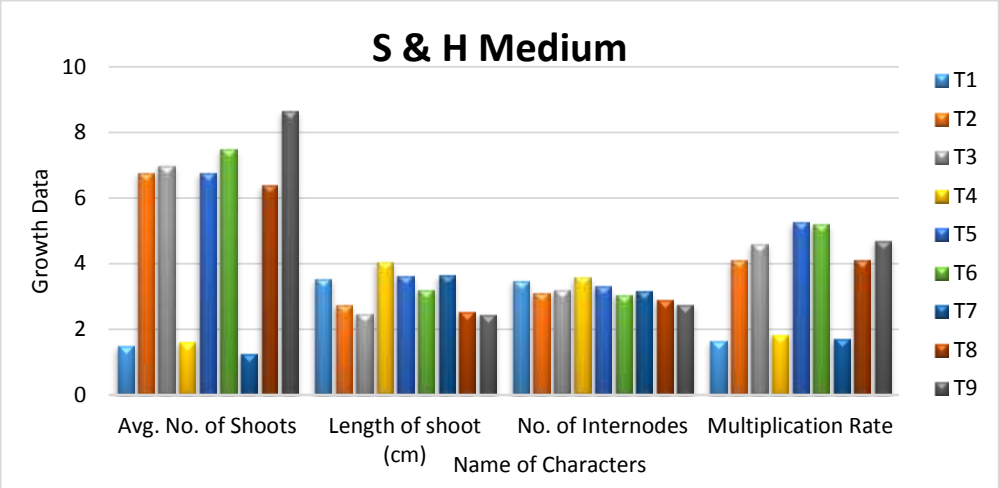


Figure 3: - *In Vitro* Growth in S & H Media Supplemented with Cytokinin and Auxin.



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