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IN VITRO PROPAGATION OF STEVIA REBAUDIANA BERTONI (A SWEETING PLANT)

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ABSTRACT

Stevia rebaudiana is a self-incompatible plant. So it cannot pollinate regularly and not produced viable seed. The seed of *Stevia* are very small and have very little endosperm. By these reasons the germinated seedling plant not survives. The objective of present investigation has been to derive the method of mass propagation by developing efficient for *Stevia rebaudiana*. The germination rate of seed can be low except half the seed slouch not to germinate. It is propagated vegitatively. Through stem cutting are used for vegetative propagation. Tissue culture plants have proven to be best planting material for stercia. Tissue culture plants of Stevia are genetically pure, free from pathogen and have exillent vigour. Systematic approaches was made in order to achieve multiple shoot induction from node of mature and juvenile stem explants of *Stevia rebaudiana* NAA (0.1 mg^{-1}) with BAP 2.0mgl^{-1}) in MS medium was found optimum for induction of multiple shoot buds (14-18) from explant. The incorporation of NAA (0.1 mgl^{-1}) in medium not caused multiplication of the shoot but also affected the subsequent rooting of the *In vitro* raised shoots of *Stevia rebaudiana*. The shoot buds excised from explant after 4 weeks were subcultured on MS medium containing NAA (0.1mgl^{-1}) with various concentrations of BAP. Amongst all the concentration of BAP tried, 0.5 - 2.0 mgl^{-1} was found the best for further multiplication of shoots. On this medium each shoot bud formed 25-28 shoots within a week. In a period of 4 weeks newly produced shoots attained a length of 2-3 cms and could be transformed for rooting. In *Stevia rebaudiana* the original mature explant produced 14-18 shoot-buds on MS + 2.0 mgl^{-1}BAP + 0.1 mgl^{-1} NAA. Each of the *In vitro* regenerated shoot buds subcultured on fresh medium proliferated into 25-28 shoots in

one week. The same explants could be subcultured on fresh medium for five times and after each subcultured it produced 20-28. In the nodal explant produced 10-12 shoots and the same explant after excision of regenerated shoots was subcultured to fresh MS medium containing 1.0 mgl^{-1}BAP + 0.1 mgl^{-1} IAA where it again produced 15-20 shoots with in a period of 10 days. This explant could be sub-cultured six times after every 25-30 days, on fresh medium. The formation of new shoots from subcultured explant and development of older shoots took place simultaneously. In *Stevia rebaudiana* the callus was raised from leaf and juvenile segments. This was achieved on MS medium containing 2.4-D (1.0-2.0 mgl-1) or NAA (1.0-5.0 mgl-1). These calluses multiplied rapidly in the same medium on surface and margins of callus. Before the formation of these structures the callus became hard. For the callus culture on MS medium containing 1.0 – 2.0 mg/lit concentration of 2,4-D was found to be best for induction of callus.The rooting of *In vitro* shoots was achieved on MS ½ strength salt medium. Various media tried for rooting of shoots of these plants were white's, B5, WP, and MS dilution of ¾. ½, ¼ salts. Although rooting was observed in all the media with or without growth regulators in *Stevia rebaudiana* rooting was obtained only on MS medium. In *Stevia rebaudiana* 6-7 sturdy fast growing roots per shoot with lateral roots were produced on medium containing 0.5 mgl^{-1}IBA+0.1 mgl^{-1} NAA. These roots grow up to a length of 5-10 cms in 5-6 weeks. This medium is optimum for ideal rooting in the aseptically grown shoots of *Stevia rebaudiana.* The MS full strength medium containing 1.0 mgl^{-1}IBA was found the best for regeneration if roots with 8-10 cms length in 6 weeks in *Stevia rebaudiana*. In *Stevia rebaudiana* strong 7-8 roots withfast growth (8-10 cms in 6 weeks) were formed from each shoot MS ½ strength salt +0.5 mgl^{-1}IBA. Lowering of agar-agar from 0.8-0.6 percent improved rooting of shoots and subsequent growth of roots. Higher concentration of agar may be inhibitory to some growth factors of cultures. The better growth of roots can be explained by easy penetration of the growing roots in the medium due to less solidification.

KEYWORDS: *In vitro,* NAA, BAP, 2,4-D, IBA,Relative Humidity (RH).

INTRODUCTION

Stevia, botanically known as *Stevia rebaudiana* Bertoni (Family- Asteraceae) is a sweet herb. A perennial herb, *Stevia* is a member of the daisy family. The leaves are mid green and intensely sweet. The compounds in the leaves are called stevioside and rebaudioside and they can be more than 200 times sweeter than sugar. The plant bears greenish cream flowers in autumn. Although *Stevia* has been in use in Asia and Europe for years, it was only in the past

couple of years that it really started to capture attention in the Indian market as a healthy alternative sweetener to sugar. *Stevia* has no calcium cyclamate, no saccharin, no aspartame, and no calories. It is safe for diabetics, as it does not affect blood sugar levels; it does not have the neurological or renal side effects associated with some of the artificial sweeteners. *Stevia* is a new crop in that is gaining very high popularity amongst all type of sweetener users as most ideal substitute for sugar. Sugar is basically a chemical that has grown in market over last many years. But in this age of changing life styles and people becoming more conscious of their health, the worldwide sugar consumption is going down and is getting replaced by low calorie sweeteners. Many of these Sweeteners are complex chemicals or many times naturals as well. *Stevia rebaudiana* Bertoni is one of 154 members of the genus *Stevia. Stevia* was first brought to the attention of Europeans in 1887 when M.S. Bertoni learned of its unique properties from the Paraguayan Indians and Mestizos (Lewis, 1992). The first reports of commercial cultivation in Paraguay were in 1964 (Katayama et al. 1976; Lewis, 1992). A large effort aimed at establishing *Stevia* as a crop in Japan was begun by Sumida (1968). Since then, *Stevia* has been introduced as a crop in a number of countries including Brazil, Korea, Mexico, United States, Indonesia, Tanzania, and, since 1990 Canada (Lee et al. 1979; Goenadi, 1983; Shock 1982; Saxena and Ming 1988; Brandle and Rosa 1992; Fors 1995). Currently *Stevia* production is centered in China and the major market is in Japan (Kinghorn and Soejarto 1985). No large scale mechanized production has been established and *Stevia* sweeteners are not yet found in mainstream food products in most countries of the world. Progress towards large scale commercialization has been slow, largely due to difficulties in producing the crop, the poor quality of *Stevia* extracts and the absence of regulatory approvals essential for *Stevia* sweeteners in the North American and European markets. *Stevia rebaudiana* Bertoni is one of 154 members of the genus *Stevia* It is a member of Asteraceae family and as such is related to sunflower, marigolds etc. *Stevia* has an alternate leaf arrangement and herbaceous growth habit with flowers arranged in intermediate heads. The flowers are small and white with a pole purple throat. The pollen can be highly allergenic. *Stevia* is self-incompatible and probably insect pollinated, the seed are small, have very little endosperm and dispersed in the wind via a hairy pappus. Propagation of *Stevia* is usually by stem cutting which root easily, but requires high labor inputs. Poor seed germination is one factors limiting large scale cultivation. The plant biotechnology is a potent methodology to enhance the formation and accumulation of desired natural plants products. The tissue culture of *Stevia rebaudiana* and its components have been studied by Miyagowa, H; Fujikowa, N; Khoda, H., Yamasaki, K; Taniguchi, K.; and Tanaka, R. (1986). Tissue

cultures of *Stevia rebaudiana* have been studied by Handro, - W; Hell,-K-G; Kerbany, -G-B (1977). Some workers have obtained phytosterol from the callus of *Stevia rebaudiana* (Nabeta, K; Kasai, T; Sungisawa, H. 1976). Tissue culture is the best option but many formers attempted to try the stem cutting method for multiplication stem cutting sometimes more expensive produce then the tissue culture since the success rate of the stem cutting establishment is very low, it takes minimum of 25 weeks for the stem cutting to develop in proper feed root for transplantation. *Stevia* is a versatile herb with incredible sweetness that can be safely used in herbal medicines; tonic for diabetic patients and also in the daily usage products, *Stevia* leaves can be used because of its anti-fungal and anti-bacterial property. Mild *Stevia* leaf tea offers excellent relief for an upset stomach. A wet *Stevia* leaf bag provides cooling effects on eyes (similar to using cucumber). The leaves effectively tighter the skin and are good for wrinkles. *Stevia* has proved to give exceptional benefits when used on blemishes, wounds, cuts and scratches. *Stevia* is helpful in weight and blood pressure management. It has also been reported that *Stevia* lower incidence of colds and flu. *Stevia* finds its use as natural sweetener, replacing the chemical sweeteners and even table sugar, the sweetness in the leaf is done to the presence of an intensive sweetening agent called stevioside and the leaf by itself is about 20 to 30 times sweeter than sugar. The leaf has stevioside of 10-12% on try weigh basis. *Stevia* is a new promising renewable raw material for the food market. The market potential for this natural sweetener is still untapped. It is estimated that about 30 million Indians are presently suffering from diabetes and it is estimated that by India's contribution to the diabetic global population would be a whopping 80 million. With such a huge share of the population being diabetic, the new ventures in the food industry are focused entirely on them. The leaves of this plant are 30 times sweeter than sugar; with zero calories where as pure extract is 300 times sweeter than sugar. This sweet honey leaf herb is likely to become the major source of high potency sweetener for the growing natural food market, in the years to come.

MATERIALS AND METHODS

(A) Collection of plant material:- In the present investigation the stem explant of nature plant *(Stevia rebaudiana)* with auxiliary and terminal buds , Nodal stem segments is used to establish the aseptic culture. The collected plant material were first washed with tap water and then washed with distilled water. After this the explant wash with 0.1% $Hgcl_2$solution which followed by several washes with water and finally with autoclaved water. After surface sterilization of explants these were inoculated aseptically on to different culture media.

(B) Culture media: -Many nutrition media or culture media are developing for tissue culture. The composition of plant tissue culture media is: inorganic salt, vitamin, amino acid, plant growth regulators, carbohydrates and the medium matrix, all the component are soluble in distilled water. Various types of media are used in tissue culture. These include MS basal medium, Gamborg's B5 medium, WS medium, Hogland nutrient medium and White medium. For preparation of medium, after mixing the salts in distilled water, the agar-agar was added in the medium. The amount of agar-agar very from 0.5-0.8 % and that of sucrose 3 to 4 % which will be used as a source of carbohydrate. The pH will be adjusted to 5.8 $\pm$ 0.2 with 0.5N KOH and 0.1N HCL solution before sterilization. 15 to 20 ml and 35 to 40 ml medium will be poured in tubes and conical flasks respectively. Finally the medium will be sterilized by autoclaving it at 15 pound per square inch (psi) pressure for 15 minutes.

(C) Inoculation:-The inoculation of plant materials will be carried out in laminar air flow cabinet under sterile condition. All appliances weresterilized and the explant after surface sterilization was inoculated vertically or horizontally on the surface of medium. The inoculated culture flasks and tubes were kept in culture room under controlled condition.

(D) Culture condition:-The culture will be maintained at the temperature of 28$\pm$ 2˚C with 14 Hrs.illumination of the light of intensity 2000 to 5000 lux and relative humidity (RH) 60 to 70 % a source of light will fluorescent tubes and incandescent bulbs. The temperature, light and RH will be varied according to the experiment.

(E) Subculture and multiplication of propagules:- After establishment of explants aseptically in culture media these were subculture either on same medium, or on the modified medium with some changes in ingredient and growth hormones like Benzyl amino purines (BAP); kinetin (Kin.); Indole-3-acetic acid (IAA); Naphthalene acetic acid (NAA); Indole-3-butyric acid (IBA); 2,4-Dichlorophenoxy acetic acid (2,4-D) and Gibberellic acid (GA_3). The subculturing was carried out in some cultures for 5-6 times to multiply them for further experiments. The tissues will be used either individually or in combinations of different concentrations.

(F) Root induction:- When the aseptic shoots will be raised insufficient numbers, these were transferred to root inducing media like Whites MS basal, MS ½ salt strength, ¾ strength and ¼ strength of MS salts. *In vitro* produced shoots will be transferred to the other media like B5

basal medium, Hoagland'smedium supplemented with vitamins, amino acids and various root inducing hormones like NAA, IAA and IBA. The physical condition, concentrations and combinations of growth regulators, vitamins, amino acids and inorganic salts of media will be changed according to need of culture.

Table – 1: Media Composition.

Element	Formulae	MS (mg/l)
MACRO-ELEMENT		
Ammonium nitrate	NH_4NO_3	1650
Potassium nitrate	KNO_3	1900
Calcium nitrate	$Ca(NO_3)_2.4H_2O$	-
Calcium chloride	$CaCl_2.2H_2O$	440
Magnesium sulphate	$MgSO_4.7H_2O$	370
Potassium sulphate	K_2SO_4	-
Ammonium sulphate	$(NH_4)_2SO_4$	-
Potassium dihydrogen phosphate	KH_2PO_4	170
Sodium dihydrogen phosphate	$NaH_2PO_4.H_2O$	-
MICRO-ELEMENT		
Boric acid	H_3BO_3	6.2
Potassium iodide	KI	0.83
Sodium molybdate	$Na_2MoO_4.2H_2O$	0.25
Cobalt chloride	$CoCl_2.6H_2O$	0.025
Manganese sulphate	$MnSO_4.4H_2O$	22.3
Zinc sulphate	$ZnSO_4.7H_2O$	8.6
Copper sulphate	$CuSO_4.5H_2O$	0.025
Ferrous sulphate	$FeSO_4.7H_2O$	27.80
Disodium salt of ethylene diamine tetra acetic acid	Na_2-EDTA.$2H_2O$	37.3
VITAMIN		
Thiamine HCl		0.1
Nicotinic acid		0.5
Pyridoxine-HCl		0.5
Glycine		2.0
Meso-Inositol		100
Folic acid		-
Biotin		-

Table – 2: Composition Of Hoagland Nutrient Solution

Macronutrients	**g/1**
$Ca\ (No_3)_2.4H_2O$	0.94
$MgSo_4.7H_2O$	0.52
KNO_3	0.66
$NH_4.H_2PO_4$	0.12
Sequestren 330 Fe	0.07
Micronutrients	**g/1**
H_3BO_3	28

$MnSO_4.H_2O$	34
$CuSo_4.5H_2O$	1.0
$ZnSo_4.7H_2O$	2.2
$(NH_4)_6Mo_7O_{24}.4H_2O$	1.0
H_2So_4 (conc.)	5.0ml

0.1 ml of micronutrients is mixed with 1 liter of macronutrients and the pH adjusted to 6.7.

RESULTS AND DISCUSSION

Stevia rebaudiana Bertoni Belonging to the family Asteraceae is one of the most valuable tropical medicinal plants. It is a source of low calorie sweetener and also used in beverage. It is a self-incompatible plant. So it cannot pollinate regularly and not produced viable seed. The seed of *Stevia* are very small and have very little endosperm. The germination rate of seed can be low except half of seed shown not to germinate. This is alternative method for multiplication of species in which propagation through seed is very poor and vegetative method of propagation is not available. In present investigation to achieve highly effective protocol for *In vitro* multiplication of *Stevia rebaudiana*.The explants showed swelling at nodal region which was followed by the emergence of shoot buds. Multiple shoot formation was achieved from pre- existing meristems of nodal region of explants. The time taken for shoot initiation from explant was dependent of growth hormones and nutrient media but the number of shoots per explant was dependent on the type and concentration of growth hormones. The juvenile and mature explants of *Stevia* were inoculated on MS medium containing BAP and kinetin alone (0.1 to 5mgl^{-1}). The number of shoots produced per explant varied in different concentrations of BAP and kinetin but the time period required for shoot initiation in all the treatment was similar (Table-3). The juvenile explants proliferated in 5 days whereas as the mature ones produced shoots in 10 days in all concentrations of BAP and Kinetin in MS medium. As the BAP concentration increased from 0.1mgl^{-1} the numbers of shoot buds were also increased as compared to control (no growth regulators). The maximum numbers of shoots (14-18) were observed on 2.0mgl^{-1} BAP. The growth of regenerated shoots on 2.0mgl^{-1} BAP was only 0.5cms. The shoot formation was also achieved from both the types of explants on kinetin (0.1 to 5.0mgl^{-1}) supplemented medium, maximum 1-2 shoots from juvenile explant and 4-5 shoots from mature explants were observed. As far as the effect of various concentrations of BAP and kinetin on shoots regeneration from both the types of explants (matured and juvenile) it was concluded that the matured explants were suitable for induction of maximum multiple shoots in both the growth regulators BAP and kinetin.

Among the various concentrations of BAP and kinetin. The BAP (2.0mgl^{-1}) was found to be more suitable for induction of maximum number of shoots in matured explants, but the concentration of BAP higher than 2.0 mgl^{-1} BAPmgl^{-1} the shoot of number was reduced. The shoots produced in MS + 2.0mgl^{-1} BAP was maximum in number but the growths of shoots were only 0.5 cms in 3-4 weeks. In order to enhance the growth of shoots several attempts have been made by incorporating different auxins (2, 4-D, NAA and IAA) with BAP 2.0mgl^{-1} in MS medium (Table-4) of all the auxins with various concentrations (0.1mgl^{-1} to 5.0mgl^{-1}). The 2,4-D gave callusing where as in IAA supplemented medium the shoot growth was not enhanced and little callusing on this medium was observed at lower end of the shoots. NAA added in the concentration ranging from 0.1mgl^{-1} to 5.0mgl^{-1} in order to achieve fast growing shoots. On the medium containing 0.1mgl^{-1} NAA with BAP 2.0mgl^{-1} the growth of shoot buds enhanced to 3-4cms within 3-4 weeks with the same number of shoots whereas the concentration of (1mgl^{-1}to 5mgl^{-1}). The 2, 4-D gave callusing where as in IAA supplemented medium the shoot growth was not enhanced and little callusing on this medium was observed at lower end of the shoots. NAA added in the concentration ranging from 0.1mg^{-1} to 5.0mg^{-1} in order to achieve fast growing shoots. On the medium containing 0.1mg^{-1} NAA with BAP 2.0mg^{-1} the growth of shoot buds enhanced to 3-4 weeks with the same number of shoots per explants. The lower concentration of NAA was ineffective for growth of shoots whereas the concentrations of NAA higher than 0.1mgl^{-1}inhabited the shoot growth and have induced callusing at the base of shoots (Table-4) Exercise shoots regenerated on MS medium containing BAP 2.0mg^{-1} and NAA 0.1mg-1were transferred on to the rooting medium individually. The IBA and NAA were used individually and in combination to induce root in the regenerated shoots. Although root induction was achieved on auxin free MS medium but these roots were of poor growth (Table-6). The effect of IBA (0.01 to 2.0mg^{-1}) was also studied which revealed that on 0.5mg^{-1} IBA root induction was 60% within 10-12 days. On the lower concentration of IBA rooting reduced to 10% and in the concentration of IBA higher than 0.5 mg^{-1} only 40% shoots was rooted with callus. When NAA in various concentration (0.01 to 1.0mg^{-1}) was incorporated with IBA. The rate of rooting was enhanced to 80% (Table-7) It was observed that a combination of IBA (0.5mg^{-1}) and NAA (0.1mg^{-1}) in MS medium was found to be optimum for achieving maximum (80%) rooting *In vitro* shoots of *Stevia rebaudiana.*

Effect of BAP/ Kinetin

At first explant wash with tap water and then treated with 0.1% $HgCl^2$ solution which followed by several washes with sterile water. After surface sterilization the juvenile and mature stem explants of *Stevia rebaudiana* were inoculate aseptically on MS medium containing BAP/kinetin alone in the concentration ranging from 0.1 to 5.0mg/l. The results obtained are presented that the number of shoots produced per explant varied in various concentrations of BAP/Kinetin but the period of time required: The juvenile explants proliferated in 5 days whereas the mature ones produced shoots in 10 days of inoculation on all the concentrations of BAP/kinetin in MS medium (Table-3). On control, only 4 shoots were produced from juvenile explants. The shoot number increased from 4 to 10 from such explants as the concentrations of BAP was raised from 0.1 to 5.0mgl^{-1}. On control, 6 shoots were regenerated from nodal region of mature explants. As the BAP concentration increased from 0.1mgl^{-1} to 5.0mgl^{-1}. The number of shoot buds per mature explant increased from 6 to 14 on BAP (1.0mgl^{-1}) 11 shoot buds were produced from mature explant. The higher concentration of BAP (5.0mgl^{-1}) inhibited the growth of shoots. The shoots produced on this medium attained 0.5cm length. The optimum concentration of BAP for induction of multiple shoot buds (14-15 per explant) was found to be 2.0 $mgl^{-1}$the shoot formation was also achieved from both the types of explants on kinetin supplemented medium. On medium containing kinetin (0.1 to 1.0mgl^{-1}) only 1-2 shoots were produced from juvenile explant. The higher concentration of kinetin (2.0mgl^{-1} to 5.0mgl^{-1}) in the medium inhibited the shoot proliferation from both the types of explant.

Effect of BAP + auxins

On the basis of previous experiments the mature stem explant was selected as appropriate explant for further experimentation as this produce maximum number of shoot buds on MS medium with BAP (2.0mgl^{-1}). The shootbuds regenerated from node of explant, however, grow poorly; they attained a length of 0.5cm in 4 weeks. In order to enhance the growth and vitality of such buds several attempts were made by incorporated different auxins. For these purpose auxins like 2,4-D/NAA/IAA was incorporated in above medium (MS + BAP 2.0mgl^{-1}). Results are presented in Table-4.

Effect of 2, 4-D

The 2, 4-D was added in the medium in concentration range of 0.01 to 1.0mgl^{-1} on 2, 4-D (0.01mgl^{-1}) containing MS medium 10 shootbuds (1cm length) were produced in 4 weeks. As

the concentration of 2, 4-D increased from 0.1 to 1.0mgl^{-1} the shoot formation was inhibited. On 2, 4-D 0.5mgl^{-1} only 6 shootbuds were produced. The 1.0 mgl^{-1} 2, 4-D in MS medium was completely inhibited shootbuds formation. On this medium vigorous callus was also induced by explant.

Effect of NAA

NAA was added to the medium in concentrations ranging from 0.1mgl^{-1} to 5.0mgl^{-1} in order to achieve healthy, fast growing and strong shootbuds. Maximum numbers of shoots (13-15) were produced on medium containing NAA (0.1mgl^{-1}). On this medium the shootbuds so produced were strong (2mm thick) and fast growing. As the concentration of NAA in the medium raised to 0.5mgl^{-1+} the number of shootbuds/explant was decreased. Beyond the 0.5mgl^{-1} NAA the number of shootbuds per explant was gradually decreased and shoot production was completely inhibited on NAA 5.0mgl^{-1}. On higher concentration of NAA vigorous callusing was also noted.

Effect of IAA

On IAA supplemented medium the number of shoots produced was less. On 0.1mgl^{-1} of IAA 12 shootbuds were produced from the explant but the growth of these shoots was per (1.6 cms in 4 weeks). As concentrations of IAA increased the number of shootbuds decreased and the explant showed callusing. These experiments proved that for induction of healthy multiple shootbuds the nature explants should be cultured on MS medium supplemented with 2.0mgl^{-1} NAA. The shoots differentiate on this medium could be used for further multiplication and rooting experiments.

Multiplication of Propagules and Repeated Sub Culturing of explant: Once the proliferation of shoots is achieved from *In vitro* cultured explants. The next step is to multiply the *In vitro* growth shoots. In *Stevia rebaudiana* during present investigation experiments were conducted to further multiply the shoot buds regenerated from stem explants. These shoot buds after 4 weeks of culture, when attained a length of 2.5cms were excised from explant and carefully subcultured to the fresh MS medium containing various growth regulators.

Effect of Cytokines' on shoot multiplication:- The shoot buds achieved from stem explants cultured on MS medium containing BAP (2.0mgl^{-1}) + NAA (0.1mgl^{-1}), were further sub cultured on the MS medium with BAP/kinetin alone. The effect of BAP/kinetin alone on shoot multiplication was studied.

Effect of BAP/kinetin:- The results obtained are reveal that in controlled medium (MS without growth regulators) about 6 shoots/explant were produced each having a length of 3cms in 4 weeks. As the concentration of BAP increased from 0.1 to 5.0mgl^{-1} the numbers of shoots produced were also increased. On MS + BAP 2.0mgl^{-1} 14 shoots were regenerated from a single bud/explant. The maximum number of shoots were produced of BAP (0.5mgl^{-1}) but on this medium shoot growth was very poor i.e. length 0.5cms in 4 weeks. The length of shoots was further decreased with the increase in the concentration of BAP from 0.5 to 5.0mgl^{-1}on BAP and 2.0mgl^{-1} the number of shoots prolifered from the bud was 14-18but their length was only 0.5cm in 4 weeks. The length of regenerated shoots was reduced to 0.26cm on MS + BAP 5.0mgl^{-1}. Thus with the increase in BAP concentration the number of shoot did not decrease considerably but the length of individual shoot was reduced significantly. On the MS medium containing kinetin.

Effect of Kinetin + NAA/IAA: -Results prove that the kinetin in combination with NAA/IAA was not found as effective as BAP for induction as well as for growth of shoots from sub-cultured shoot buds. The kinetin (1.0mgl^{-1}) with NAA/IAA individually (0.1 to 5.0mgl^{-1}) was incorporated in the medium. On lower concentration of kinetinup to 0.5mgl^{-1} with NAA/IAA (0.1mgl^{-1}) only 8 shoots were produced. As the concentration of kinetin increased to 2mgl^{-1} with increased concentrations of NAA/IAA (1.0mgl^{-1}) the number of shoots/bud decreased and the excessive callusing occurred. The length of shoots on higher concentrations of kinetin and auxin was reduced to 0.2cm in 4 weeks.

Callus Culture

Different types of explants namely, leaf segments, Juvenile and mature stem segments were cultured on MS medium for induction of callus. These were inoculated aseptically on MS medium containing NAA/IAA in the concentration ranging from 0.1 to 5.0mgl^{-1} 2, 4-D being stronger auxin was added in the medium in lower concentration (0.01 to 2.0mgl^{-1}). Results are presented in Table-5.

Effects of 2, 4–D:-On MS medium +0.01mgl^{-1} 2,4-D caused callusing of leaf segments and juvenile explants in about 10 days. The leaf and juvenile explants produced moderate and vigorous callus whereas only little callus developed from the mature explants. On MS + 0.5 to 2.0mgl^{-1} of 2, 4-D vigorous callus was produced from the explants. The callus turned green under light intensity of 2000-2500 lux.

Effectof NAA/IAA:-On MS + NAA/ IAA (0.1 to 1.0mgl^{-1}) little callus was developed from leaf segments and juvenile explants but mature explants did not produce callus. On higher concentration of NAA/IAA (2.0 to 5.0mgl^{-1}) little callus developed than the cut ends of the mature explants.

Subculturing of Callus:-The callus produced from leaf segments was removed from explant under aseptic conditions and subcultured on the MS medium containing 2, 4-D (0.01, 0.025, 0.05 and 0.1mgl^{-1}).These cultures were subcultured on to MS medium with above mentioned growth regulators. On MS medium with 2, 4-D (0.01 to 0.025mgl^{-1}), MS + NAA/IAA (2 to 5.0mgl^{-1}) these cultures were multiplied rapidly. After two weeks these cultures were subcultured to the MS medium containing BAP/Kin (0.1 to 5.0mgl^{-1}) or 5-methyl tryptophan (0.1 to 5.0mgl^{-1}). These cultures showed excessive browning even with little disturbance of injury.

Rooting of Cultured Shoots

Effect of MS + IBA on rooting:-The excised shoots produced on MS medium containing BAP (0.5mgl^{-1}) and NAA (0.1mgl^{-1}) or on BAP (0.5mgl^{-1}) alone were transferred on rooting medium. The medium on which the multiple shoots were produced showed marked influence on subsequent rooting behavior of such shoots on root induction medium. The requirements for induction of roots are different for the shoots originally grown on MS + 05mgl^{-1} BAP (SB type). The SB type shoots were rooted only when IBA was added to the medium while the SBN type of shoots could also be rooted only when IBA was added to the medium while the SBN type of shoots could also be rooted on auxin-free medium. Although roots produced on auxin-free MS medium were not as effective as produced on IBA containing medium. The rooting of SBN or SB types of shoots was also influenced by various concentrations of IBA. In this experiment the effect of various concentrations of IBA on SBN and SB types of shoots, was studied. The results obtained are presented in Table-6. These reveal that the rooting of SB type shoot was influenced by concentration of IBA. On lower concentration of IBA (0.01 and 0.1mgl^{-1}) these shoots did not root and could be rooted only on medium containing 0.5 mgl^{-1} to 2.0mgl^{-1} IBA. However, even on these concentrations more time was required (20-25 days) for rooting. The roots so produced grow poorly and were weak. On the other hand 10% of SBN types of shoots were rooted on auxin free medium in about 15-20 days though the growth of roots was poor. Therefore, IBA was incorporated in the medium. As the concentration of IBA increased from 0.0 to 0.5mgl^{-1} the percentage of rooting in SBN

type of shoots was rooted on medium containing IBA 0.5mgl^{-1}. On further increase in the concentrations of IBA rooting could be achieved (up to 2.0mgl^{-1} IBA) beyond this rooting was inhibited and excised shoots produced callus. Thus for good rooting IBA 0.5mgl^{-1} was optimum for SBN types of shoots. The roots so produced attained 0.1cm thickness and 4-5cms length in 4 weeks. The plantlets so developed were however, showed decline in growth. The natural propagation of this plant is very poor.

Effect of NAA:-The SBN type shoots were transferred to the MS medium supplemented with various concentration of NAA ranging from 0.1 to 5.0mgl^{-1}. The results are presented in Table 7. In control (MS without auxin) only 10% shoots were rooted in 20-25 days. The roots so produced grow 3cms in 4 weeks. As the concentration of NAA is increased in medium from 0.1 to 0.5mgl^{-1} the percentage of rooting and number and length of roots/shoot were also enhanced. 60% shoots were rooted on MS + NAA (0.5mgl^{-1}) but on this medium callus was produced at cut end of the shoots. Further increase in the concentration of NAA further enhanced the percentage of rooting. On higher than 2mgl^{-1} NAA the root formation was completely inhibited. On this medium vigorous callus was produced. On 0.1mgl^{-1} NAA 15% of shoots rooted but there was no callusing from the ends of shoots.

Effect of MS + IAA:-IAA was added in MS medium in the concentration from 0.1 to 5.0mg/lit. The rooting was not observed at lower concentration of IAA and at higher concentration (1 to 5mg/lit) callusing occurred.

Combined effect of IBA + NAA:-Though root induction was achieved on MS medium containing IBA/NAA separately, but callusing on cut end of shoot and on surface of the roots so produced was observed. The vigorous callusing caused reduction in the growth of shoots and roots. Therefore a combination of IBA + NAA was tried for effective rooting of *In vitro* produced shoots subsequent growth of plantlets the shoots when attain a length of 2-3 cms were transferred to rooting medium containing IBA (0.1 to 2.0mg/ lit) with NAA (0.01 to 1.0mg/lit). The results are presented in Table-7. It shows that as concentration of NAA was increased from 0.01mg/lit to 0.5mg/lit with keeping the concentration of IBA(0.1mg/lit.) constant, the percentage of rooted shoots was increased and also the time period taken from the root induction was reduced. When the IBA was increased to 0.5 mg/lit and with it various concentration (0.01 to 0.5mg/ lit) of NAA were added. The percentage of rooting was further increased on 0.5mg/lit IBA with 0.1 mg/lit NAA about 80% of shoots were rooted on this medium each shoot produced 6-7 main roots, these roots grew 9-11cms. In 4 weeks. Roots

also produced laterals when concentration of IBA increased to 1.0mg/lit and 2.0 mg/lit in combination with NAA (0.1 mg/lit or 0.5mg/ lit) the percentage of rooting and number of roots per shoot decreased. On these combinations callus was produced form the cut ends of shoots. At higher concentration of IBA and NAA the surface of roots. So produced splitted up and cottony callus developed.

Effect of different media:-Once the combination of growth regulators for optimum and effective rooting was standardized further experiments were conducted to obtain the suitable salt medium for rooting. The shoots were transferred to the different media containing IBA (0.5mg/lit) with NAA (0.1mg/lit). The results obtained show that among all the media tested the MS full strength salts medium is the most suitable for rooting of shoots and subsequent growth of plantlets of *Stevia rebaudianaIn vitro*. MS full strength salts medium is the most suitable for rooting of shoots and subsequent growth of plantlets of *Stevia rebaudiana*.

Table No. – 3: Effect of BAP and Kinetin on Shoot Induction of Explants of Stevia Rebaudiana.

Concentration (mgl^{-1})	No. of day required for shoot initiation		Morphogenetic response of shoots produced by explant in 4 weeks			
			Juvenile explant		Mature explant	
	Juvenile explant	Mature explant	Mean No. of Buds ±BD	Mean length (cms) ± SD	Mean no. of Buds ± SD	Mean length (cms) ± SD
Control	5	10	4 ± 0.80	3.0 ± 0.25	5.6 ± 0.94	3.8 ± 1.00
BAP						
0.1	5	10	4±0.80	3.0±0.32	5.8±1.34	3.6±1.0
0.5	5	10	5±0.63	3.5±1.41	6.7±0.94	3.1±1.00
1.0	5	10	5±0.55	3.0±1.00	8.1±0.82	3.0±0.92
1.5	5	10	6±1.21	3.0±0.92	10.8±0.92	2.0±0.52
2.0	5	10	5±0.92	4.0±1.24	14.2±1.52	0.5±0.01
2.5	5	10	4.5±2.50	2.0±0.95	10.6±2.21	0.4±0.03
Kinetin						
0.1	5	10	2±0.52	3.0±0.52	3.0±1.22	3.1±1.20
0.5	5	10	1±0.12	2.5±1.21	3.0±0.98	2.6±0.52
1.0	5	10	2±0.42	1.0±0.32	4.0±1.21	2.7±0.23
1.5	5	10	-	-	3.0±1.52	1.9±0.22
2.0			-	-	-	-
2.5			-	-	-	-

Each value represents the mean ± SD of 5 replicates

Table No. – 4: Effect of BAP + Auxins (2,4-D, NAA, IAA) on shoot formation the explants of *Stevia rebaudiana* on MS Medium.

BAP (2.0 mgl^{-1}) + auxins conc. (mgl^{-1})	Mean number of shoots ± SD	Mean length of shoots ± SD (cms)	Callusing
2,4-D			
0.01	10.20±1.49	3.19±1.16	+
0.1	8.60±2.14	2.11±0.87	++
0.5	6.22±0.92	2.00±0.51	+++
1.0	-	-	+++
NAA			
0.1	13.21±2.51	3.51±1.28	-
0.5	11.41±2.11	2.12±0.98	+
1.0	10.27±1.47	2.27±1.92	++
2.0	10.16±2.01	1.92±0.45	++
5.0	8.66±1.82	0.52±0.13	+++
IAA			
0.1	12.11±2.19	1.66±0.58	-
0.5	11.44±1.99	1.21±0.46	-
1.0	10.27±1.92	2.11±1.62	+
2.0	8.17±2.51	3.60±0.92	++
5.0	4.40±1.78	2.50±0.86	+++

- = no callus; + = little callus; ++ = moderate callus; +++ = vigorous callus.

Each value represents mean ± SD of 5 replicates.

Table no. – 5: Effect of auxins on callus formation in different explants of *Stevia rebaudiana.*

Auxin concentration (mgl^{-1})	Response of explants for callusing		
	Leaf segments	Juvenile explants	Mature explants
2,4 – D			
0.01	+	+	-
0.1	+	+	+
0.2	+	+	+
0.5	++	++	++
1.0	+++	+++	++
2.0	+++	+++	++
NAA			
0.1	+	-	-
0.5	+	-	-
1.0	+	+	-
2.0	++	++	+
5.0	+++	++	+
IAA			
0.1	+	-	-

0.5	+	-	-
1.0	++	+	-
2.0	++	++	+
5.0	+++	+++	+

- = No callusing; + = little callusing; ++ = Moderate callusing; +++ = vigorous callusing.

Table no. – 6: Effect of IBA on rooting of *in vitro* shoots of *Stevia rebaudiana*.

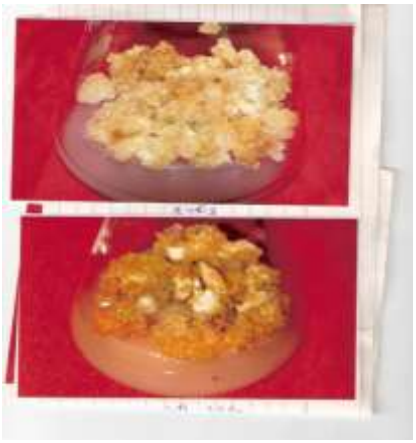
MS concentration Mgl^{-1}	Rooting responses of shoots		Callusing
	Days of root Induction	Percentage of rooting	
IBA			
0.0	15-20	10	-
0.01	12-15	10	-
0.1	12-15	25	-
0.5	10-12	60	+
1.0	10-12	50	++
1.5	8-10	40	++
2.0	8-10	45	+++

- = no callus; ++ = moderate callus; + = little callus; +++ = vigorous callus.

Table no. – 7: Combined effect of IBA + NAA on rooting of cultured shoots of *Stevia rebaudiana.*

Concentrations (mgl^{-1}) + MS medium	Morphogenetic response of roots after 4 weeks			
	Days of rooting	Percentage of rooting	Mean length of roots/shoots ±SD	Mean number of roots±SD (cms)
IBA+NAA				
0.0+0.0	20-22	10	1.46±0.48	1.82±0.61
0.1+0.01	20-22	10	1.11±0.28	1.72±0.55
0.1+0.05	20-22	15	1.62±0.52	1.64±1.68
0.1+0.1	18-20	20	2.80±0.67	2.68±1.12
0.1+0.5	18-20	20	2.62±0.42	2.86±1.11
0.5+0.01	10-15	40	3.80±1.21	2.42±0.61
0.5+0.05	10-15	50	2.66±0.84	4.85±1.21
0.5+0.1	10-12	80	6.33±1.54	9.54±2.51
0.5+0.5	10-12	60	4.45±1.44	5.55±1.46
1.0+0.01	10-15	50	2.62±1.11	3.89±1.11
1.0+0.1	10-15	50	2.12±0.62	3.56±0.20
2.0+0.1	12-15	60	3.12±0.89	1.52±0.51
2.0+0.5	12-15	40	2.61±0.58	1.12±0.45

Each value represents mean ± SD of 5 replicates.





CONCLUSION

Plant tissue culture techniques are a general technique of regeneration of those plants which cannot regenerate or propagated by natural procedure. *Stevia rebaudiana* is a source of low calorie sweetener and also used in beverages. It is a safe tonic for diabetic patients and also in the daily usage products, *Stevia* leaves can be used because of its antifungal and antibacterial property. *Stevia rebaudiana* is a self-incompatible plant. So it cannot pollinate regularly and not produced viable seed. The seed of *Stevia* are very small and have very little endosperm. By these reasons the germinated seedling plants not survive. The objective of present investigation has been to derive the method of mass propagation by developing efficient for *Stevia rebaudiana*. The germination rate of seed can be low except half the seed slouch not to germinate. It is propagated vegitatively. Through stem cutting are used for vegetative propagation. Tissue culture plant has proven to be best planting material for stercia. Tissue culture plant of *Stevia* is genetically pure, free from pathogen and has exillent vigour. One of the most important factors in obtaining multiple shoots is a choice of explant. In deciding on a suitable explant for regeneration of shoots *In vitro* several factors should be considered (Murashige, 1974). These are: - The organ that is to serve as tissue source, the physiological and ontogenetic age of the organ, the season in which the explant is being obtained, the size

of explant, and the overall quality of form which explants are to be obtained. Systematic approaches was made in order to achieve multiple shoot induction from node of mature and juvenile stem explants of *Stevia rebaudiana* NAA ($0.1mg^{-1}$) with BAP $2.0mgl^{-1}$) in MS medium was found optimum for induction of multiple shoot buds (14-18) from explant. The incorporation of NAA ($0.1mgl^{-1}$) in medium not caused multiplication of the shoot but also affected the subsequent rooting of the *In vitro* raised shoots of *Stevia rebaudiana*. The shoot buds excised from explant after 4 weeks were subcultured on MS medium containing NAA ($0.1mgl^{-1}$) with various concentrations of BAP. Amongst all the concentration of BAP tried, 0.5 - $2.0mgl^{-1}$ was found the best for further multiplication of shoots. On this medium each shoot bud formed 25-28 shoots within a week. In a period of 4 weeks newly produced shoots attained a length of 2-3 cms and could be transformed for rooting.In *Stevia rebaudiana* and several crops of shoots were produced by repeatedly subculturing of the original explant which remained after excision of developed shoots each time. In *Stevia rebaudiana* the original mature explant produced 14-18 shoot-buds on MS + $2.0mgl^{-1}$ BAP + $0.1mgl^{-1}$ NAA. Each of the *In vitro* regenerated shoot buds subcultured on fresh medium proliferated into 25-28 shoots in one week. The same explants could be subcultured on fresh medium for five times and after each subcultured it produced 20-28. In the nodal explant produced 10-12 shoots and the same explant after excision of regenerated shoots was subcultured to fresh MS medium containing $1.0mgl^{-1}$ BAP + $0.1mgl^{-1}$ IAA where it again produced 15-20 shoots with in a period of 10 days. This explant could be sub-cultured six times after every 25-30 days, on fresh medium. The formation of new shoots from subcultured explant and development of older shoots took place simultaneously. In the present investigations too, it has been possible to subculture original explants of repeatedly. In each subculture the better yield of crop of shoots plants. In our laboratory the techniques of repeated subculturing has been applied for several species. In *Stevia rebaudiana* the callus was raised from leaf and juvenile segments. This was achieved on MS medium containing 2.4-D (1.0-2.0mgl-1) or NAA (1.0-5.0mgl-1). These calluses multiplied rapidly in the same medium on surface and margins of callus. Before the formation of these structures the callus became hard. For the callus culture on MS medium containing 1.0 -2.0mg/lit concentration of 2, 4-D was found to be best for induction of callus. It is well established fact that growth regulators exert carry-over effect on the tissues when these are transferred from one medium to the other or from higher concentration of growth regulators to lower ones. They also affect subsequent behavior of tissues *In vitro* (Hu and Wang, 1983). Nemeth (1986) has suggested that an optimal endogenous auxin-cytokinin balance might be established for the root formation. During *In vitro* studies of

Stevia rebaudiana it is recorded that if shoot differentiation was obtained on a medium with cytokinin alone, rooting of such shoots was difficult to achieve and if a few shoots could be rooted, the subsequent growth of plantlet. In fact such plantlet could not survive for more than 1-2 weeks *in vitro*. Incorporation of auxins (NAA) in concentration of 0.1/0.5 mgl^{-1} in shoot differentiating medium facilitate rooting of shoots. The growth rate of such shoots was higher after rooting. About 80 percent of shoots, differentiated on NAA containing medium, were rooted while only 20 percent shoots were rooted if these shoots differentiating on cytokinin alone or with IAA containing medium. The shoots obtained from various sources were successfully rooted. This was possible with extensive experimentation. Although rooting was observed in all the media with or without growthregulators in *Stevia rebaudiana* rooting was obtained only on MS medium. In *Stevia rebaudiana* 6-7 sturdy fast growing roots per shoot with lateral roots were produced on medium containing 0.5mgl^{-1} IBA+0.1mgl^{-1} NAA. These roots grow up to a length of 5-10cms in 5-6 weeks. This medium is optimum for ideal rooting in the aseptically grown shoots of *Stevia rebaudiana*. The MS full strength medium containing 1.0mgl^{-1} IBA was found the best for regeneration if roots with 8-10 cms length in 6 weeks in *Stevia rebaudiana*. In *Stevia rebaudiana* strong 7-8 roots with fast growth (8-10cms in 6 weeks) were formed from each shoot MS ½ strength salt +0.5 mgl^{-1} IBA. Lowering of agar-agar from 0.8-0.6 percent improved rooting of shoots and subsequent growth of roots. Higher concentration of agar may be inhibitory to some growth factors of cultures.

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