STUDIES ON *IN VITRO* PROPAGATION OF STEVIA
(*Stevia rebaudiana* Bertoni.)

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IN

PLANT PHYSIOLOGY AND ECOLOGY

BY

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ANAND – 388 110
GUJARAT (INDIA)

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C E R T I F I C A T E

This is to certify that the thesis entitled **"Studies on *in vitro* propagation of stevia *(Stevia rebaudiana* Bertoni.)"** submitted by **Mr. Balram Kishore** in partial fulfillment of the requirements for the degree of **M. Sc.** in **Plant Physiology and Ecology** of the Anand Agricultural University is a record of bonafide research work carried out by him under my guidance and supervision. The thesis has not previously formed the basis for the award of any degree, diploma or other similar title.

Place: Anand **(SUBHASH N.)**

Date: **Major Advisor**

STUDIES ON *IN VITRO* PROPAGATION OF STEVIA

(*Stevia rebaudiana* Bertoni.)

Name of the student **Major Advisor**

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ABSTRACT

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 2025 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 would be entirely focusing on them. Increased availability of raw materials for production of steveoside is a challenge for the industry in future. Uniform disease free planting material is the prime requirement to the stevia growers. Tissue culture or micropropagation of stevia has already been shown to offer considerable advantages as compared to traditional propagation techniques for improving the production of selected variety. An investigation was undertaken to study the physiological aspects of *in vitro* propagation stevia. The experiment was conducted at Plant tissue culture laboratory, Department of Agricultural Botany, B. A. College of Agriculture, Anand Agricultural University, Anand during 2004 - 2007.

Various experiments were conducted to identify the best sterilization agent for surface sterilization of explant, to study the effect of different media and phytohormone combinations for establishment, response of different phytohormone on shoot development, effect of different nutrient media on multiplication and rooting, response of different growth regulators for *in vitro* rooting, various physiological factors affecting micropropagation of stevia and establishment of plants during hardening.

Different morphological characters or parameters *viz.* length of shoots, shoots per explants, number of nodes per shoot, days to root initiation, number of roots per shoot, length of roots, number of survived plants showed significant variation for various physiological aspects studied.

Combinations of antibiotics / bacteriocides and antifungal agents were found to eliminate surface contaminants satisfactorily. Higher percentage (60 to 80 %) of establishment and growth of cultures on full strength MS medium was observed than other media tried whereas, the range of the percentage of establishment varied slightly within various light and dark period treatments.

For multiplication, the best response was observed with 2.0 mg^{-1} of BA. Further improvement in multiple shoot formation and development was not observed when combinations of cytokinins were tried. The explants grown on MS medium showed fast bud sprouting but further growth was slower. At half and full strength also MS medium was found superior to other media. Cultures grown on ¾ strength medium showed maximum multiplication and development. Shoot initials isolated from the terminal / upper most position showed overall better multiplication and development with the highest number of shoots, more number of nodes and higher

number of leaves along with higher length of shoots. Total number of shoots, nodes and leaves were increased as the number of nodes was increased in explant.

Variation in light intensity showed significant variation for multiplication of shoots and highest number of nodes and leaves. The duration of light/dark periods were also found to exert marked effects on multiplication and development.

Subculture passages (up to sixth) were also affected the morphogenic potential of plantlets. Overall frequency of multiple shoot formation and development showed variation under the effect of different sources of carbon/ sugars. Optimization of inoculation density showed satisfactory multiplication and development with lower requirement of medium per explant.

90 to 100 percent rooting was obtained in all the MS medium based treatments. The time taken for root initiation, number of roots, length of roots and presence of callus were significantly influenced by different concentrations and type of auxins. Improvement in root formation was observed when combinations of auxins were tried. Stimulation of root growth and development was found to be influenced by the number of nodes in shoot explant. Incorporation of activated charcoal in the medium resulted in the elongation of roots. Cocopeat based potting mixtures significantly (up to 76%) increased the survival of *in vitro* raised plants during hardening. Field survival of tissue culture raised plants was found quite satisfactory.

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Anand **(Balram Kishore Singh)**

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Abbreviation	Expended
°C	Degree Celsius (Temperature)
2-iP	6-(g,g-dimethylallylamino)-purine or N6-(2-isopentyl)-adenine.
AC	Activated charcoal.
ANOVA	Analysis of variance
B5	Gamborgs Medium (1968).
BAP	N-6 Benzylaminopurine.
BM	Basal medium.
g	Gram
IAA	Indolyl-3-acetic acid.
IBA	Indolyl-3-butyric acid.
KIN/Kn	Kinetin, 6-furfurylaminopurine.
mg	Milligram
min.	Minute
ml	Milliliter
MS	Murashige and Skoog's Medium (1962).
NA	Nutrient agar.
NAA	1-Naphthaleneacetic acid.
TDZ	Thidiazouron, N-phenyl-N'-1,2,3-thidiazol-5- ylurea.
µE	Microeinstein.
µM	Micromolar
Zea/Zn	Zeatin

INTRODUCTION

The number of diet conscious people and diabetic patients is increasing day by day. Consequently, the worldwide demand for highly potential alternative non-caloric natural sweeteners is expected to increase. The sweet herb stevia synthesizes such an alternative ent-Kaurene glycosides of the diterpenes steviol, which are 450 times sweeter than sucrose.

Stevia (*Stevia rebaudiana* Bertoni) of family Asteraceae is an important medicinal crop which is grown annually as a summer crop in cold climatic regions and perennially in the tropical regions of the world.

The established uses for stevia products cover all those of artificial low-caloric sweeteners as well as most other purposes in which common sugar can be used (Brandle, 1998). It is primarily used as a sweetener to enhance the palatability of food and beverages. Unlike aspartame, Stevia sweeteners are heat stable, acid stable and do not ferment, which make them suitable for use in wide range of products including baked/cooked foods (Martelli *et al.*1985*)*.

Stevia products are now used for recovery from diabetes and to regulate blood pressure. It is also used in herbal and medicinal preparations related to skin care, anemia, mouth diseases and microbial infections (Takhashi *et al.* 2001). Stevia may also be used in production of the plant growth regulator, GA_3 (Alves and Ruddat, 1979).

The possibility of adoption of Stevia products as alternative to common sugar and artificial medicinal sweeteners has made its cultivation economically attractive to the extent that several countries now started commercial cultivation including Paraguay, Japan, China, Canada, Australia, and America. In India, recently, its limited

cultivation has been started in Gujarat, Maharastra, Karnataka, Uttranchal, Kerala and Tamil Nadu etc. on experimental basis as the current domestic consumption of Stevia plants has increased more than 630 thousand plants per year (Anonymous, 2005).

Stevia propagation is done conventionally by seeds, cuttings, and clump division or by division of mother plants (Tamura *et al*, 1984, Carvalho and Zaidan 1995).

Propagation by seeds is not efficient because of low seed viability, self incompatibility of flowers and high heterozygosity (Oddone, 1999). Moreover, wide random variation of several important characters, such as glycoside content was observed among plants obtained by sexual reproduction (Miyazaki and Wantanabe, 1974). Propagation by clump division and by cutting resulted into low number of new plantlets from donor plant and large numbers are obtained only after long period of time with high labour input.

Improvement through breeding and selection programmes was based on phenotypic selection for total glycosides in stevia leaves, which is heavily influenced by environmental conditions such as season, soil and weather. It has been also found that the yield of sweetening compounds vary according to the method of propagation and agronomical practices. More importantly, proper selection is required relatively late in the growing season year after year and quantification/qualitative analysis are based on high pressure liquid chromatographic techniques. As a result, the whole process becomes very expensive, time consuming and relatively inconvenient (Brandle and Rose, 1992).

On this account, cloning of elite plants by tissue culture methods seems to hold considerable advantages over conventional techniques as it leads to production of homozygous and homogenous population in very short period of time

(Handro *et al,* 1988). Moreover, the outcome of the same may serve as source for the exploration in the area of in *vitro* generated variability and *in vitro* selection for new stevia genotypes in future.

Although, considerable thought has been given to direct/indirect organogenesis or somatic embryogenesis from different explants viz., roots, stems and leaves (Ferreira and Handro, 1988), there are very few reports available regarding the multiplication through axillary shoot proliferation which has been found to be simple and better method in other members of Asteraceae family. These studies are also found to be restricted in the sense of using bioreactors which has been shown to be suitable only for biomass production but not for stevioside production (Nepavim and Vanek, 1998) and very little attention has been given to the explanation of surface sterilization of explants, culture establishment and hardening of tissue culture raised plants. Moreover, there is complete lack of detailed reports related to physiological aspects of Stevia micropropagation so far.

Thus the present investigation was undertaken with the following objectives:

a) To identify the best explant sterilization agent to initiate axenic cultures

b) To study the effect of different factors on culture establishment

c) To study the response of different PGRs on shoot multiplication and development

d) To study the factors affecting rooting

e) To study the various physiological factors affecting *in vitro* propagation of stevia

f) To study the parameter affecting *in vitro*/*ex vitro* hardening of tissue culture raised plants and its establishment in the field.

REVIEW OF LITERATURE

The literature pertaining to stevia tissue culture has been reviewed as follows:

2.1. Surface sterilization experiments

2.1.1. Surface sterilization by $HgCl_2$

Mercuric chloride and its soluble salts are reported as efficient sterilants (EH and S Rev. 2005). Tadhani *et al.* (2006) reported 0.1 % $HgCl_2$ treatment for i.e. 2 min. after alcohol pretreatment for eliminating surface contaminants of stevia. However, the use of $HgCl_2$ treatment for longer exposure period of > 5 min. have been also reported (Joi *et. al.*, 2003).

2.1.2. Surface sterilization by antibiotics and antifungal agents

Kulkarni (2000) reported that the explants collected during Monsoon season resulted in higher fungal contamination. Kanamycin is generally used as a selective sterilant against microbial contaminants (Mohammed *et. al.*, 2005). Mitra and Pal (2007) reported that the treatments based on bavistin were less harsh to the explants and showed decrease in fungal contamination.

2.1.3. Sequential surface sterilization:

The various reports suggested single sterilant for eliminating contaminants during stevia surface sterilization (Miyagawa *et. al.,* 1986; Nepavim and Vanek,1998). Phillips *et. al.*, (1981) reported the incompatibility or indiscriminate combinational problems when Bavistin and other antibiotics were used together.

However, Kulkarni (2000) reported the complete elimination of surface contaminants in *Taxus* spp. by a combination of antifungal and antibacterial agents along with $HgCl_2$.

2.2. Culture Establishment stage:

2.2.1. Effect of different basal media on establishment and growth:

Amazaling *et. al.* (1992) suggested that the inorganic ions of nutrient medium participate in regulatory processes and therefore attributed to promotory effect on establishment. Ibughin *et. al.* (2003), reported the use of MS medium (1962) supplemented with a maximum of 0.6 mgl^{-1} IAA and 0.6 mgl^{-1}BA for obtaining higher growth rate of stevia stemlets. Full strength MS medium has been reported as a better option for establishment of most of the plants belonging to Asteraceae family including stevia (Bondarev *et. al*., 2003). However, Better establishment and initiation of stevia cultures were also observed on half strength MS medium (Nepavim and Vanek, 1998) whereas, Miyagawa *et. al.* (1986) reported better establishment of stevia cultures on B_5 medium (1965) supplemented with BA and NAA. Khetrapal *et al.* (1999) reported that ethylene evolution was more in B_5 medium than in MS medium while, Salisbury and Ross (2003) found that the bud development or shoot elongation was ceased due to the evolution of ethylene.

2.2.2. Effect of light and dark periods on establishment:

Aswath and Biswas (2001) suggested that the illumination given at the end of the culture period may result in increased growth and development of explants. Ruffani and Massabo (2005) reported better shoot establishment in light than in

dark whereas, Parthasarthy *et al.* (2001) described the requirement of initial dark condition for only shoot emergence while further growth of Gerbera observed under 12 hrs. light regime. Contrary to this, Fotopoulos and Sotirophoulous (2007) suggested initial dark condition followed by light regime for better establishment. Initial continuous light condition resulted in mobilization of food material as a consequence of renewed enzymatic activity (Stiles and Cocking, 1969). Darkani *et. al.* (2005) reported better growth under dark conditions.

2.3. Multiplication stage:

2.3.1. Effect of different cytokinins on development of shoots:

Patil *et al*.(1996) reported the multiplication of shoots from axiliary buds as well as meristem of branches. They also reported the use of MS medium supplemented with 0.5 mgl^{-1}IAA and 10 mgl^{-1}GA for higher number of multiple shoots. Meancacimei *et al.* (1997) found that zeatin was more effective than BA or 2iP in terms of mean shoot proliferation in Olive micropropagation. It has been observed that the zeatin was converted into ribotide within 10 hrs due to cleavage of the side chains and is affected differentially by the cytokinin oxidase (Bhojwani, 1990).

Contrary to this, Tavazza *et al.* (2004) observed better shoot proliferation together with good quality of shoots of *Globe artichoke* (Asteraceae) using 2 mgl^{-1} Kinetin while, BA at 0.8 mgl^{-1} to 2.0 mgl^{-1} induced small shoots. Morini *et al.* (2003) also reported better growth of stevia shoots by replacing BA with Kn in the medium. Further, the internodes were generally fairly pronounced on Kn than BA supplementation. In line with this, Mitra and Pal (2007) reported, Kn as more efficient in producing higher number of healthy shoots than with BA. Miyagawa *et al*. (1986)

initiated multiple shoot culture from the shoot tips of *Stevia rebaudiana* on MS medium containing Kinetin (10^{-5} M).

Joi *et al.*(2003) observed the maximum number of shoots of stevia induced with 2.0 mgl^{-1}BA and 0.1 mgl^{-1} Thidiazuran supplemented with MS medium, while the maximum elongation of shoots were achieved with 2.0 mgl^{-1}BA and 0.1 mgl^{-1} TDZ with 30 gl^{-1} of maltose. Erdag and Emek (2005) reported highest number of shoots per explant on medium supplemented with very low level of TDZ (0.005 mgl^{-1}).

However, the findings of Mandal and Parthiraj (2006) revealed maximum growth when MS medium was supplemented with 2.0 mgl^{-1} BA alone. Increasing or decreasing the BA concentration resulted in decreased bud sprouting. Tadhani *et al.* (2006) also reported better performance of BA than Kn at various concentrations for multiplication of stevia shoots. They reported induction of shoots on 0.6 mgl^{-1} BA while maximum number of shoots was produced with 4 mgl^{-1} on MS medium.

2.3.2. Effect of combination of BA and Kinetin:

Outgrowth of axillary buds is well correlated with the cytokinins (Mohammed *et. al.,* 2005). It has been suggested that cytokinins independently regenerate the growth of axillary buds (Sato and Mori, 2001). Baskaran and Jayabalan (2005), showed that lower level of BA and higher level of Kn was beneficial in *Eclipta alba.* However, Kaminek *et al.,* (2005) found that at higher cytokinin concentration, over expression of the activity of enzymes decreased the bud growth.

3.2.3. Effect of different media on multiplication:

Most of the reports on stevia showed satisfactory response of shoots on MS medium (Toma *et al.,* 2000; Tadhani *et al.,* 2006; Ibughin *et al.,* 2003). However, Miyagawa *et al.* (1986) preferred B_5 medium for tissue culture of stevia, whereas LS medium was often used for organogenesis in stevia (Ferreira and Handro 1987;

Tamura *et. al.,* 1984). Use of Nitsch and Nitsch medium was reported previously by Patil *et. al.* (1996); while White's medium was also found to give comparable results in a study conducted on stevia micropropagation by Tamura *et. al*. (1984) which might have been due to its low concentration and number of salts (Razdan, 1990) whereas Nitsch and Nitsch medium contains higher Zn^{3+}, $H_3BO_3^-$ and lower Mg^+. (Hughes, 1981). Ramakanthan and Jasrai (1997) observed mobilization and rearrangement of Zn^{+++} and Mg^{++} ions during sprouting of buds. Vidalee (1995) suggested that B_5 medium can be used where higher nitrogen share (12.50) of NO_3^-:NH_4^+ ions are required than MS or LS medium (NO_3^-:NH_4^+ = 1.91).

Hughes (1981) obtained higher average length of plants on LS medium and concluded that the higher level of thiamine and exclusions of glycine and niacine could be the reason behind observed growth. Latha and Mukundan (2003) regenerated stevia shoots by culturing the shoot apices, nodal and leaf explants on MS medium supplemented with BA (8.8µM) and IAA(5.71µM). However, MS or LS medium lacks or is lowers on Na^+, Al^{3+} and Ni^{2+} (Amirouche *et al.,* 1985). George and Tripepi (2002) reported stunting of shoots and dieback of growing apices due to calcium deficiency in *Lewsia* cotyledons.

2.3.4. Selection of best medium for multiple Shoot formation:

Recent reports on stevia tissue culture (Mitra and Pal, 2007; Tadhani *et al.,* 2006; Mandal and Parthiraj, 2006) showed that the MS medium was preferred for multiple shoot formation. LS medium was used for organogenesis studies in stevia by Tamura and Handro (1988) whereas, lower salts containing medium viz, B_5 medium have been used for tissue culture of several crops (Rout *et al.,* 2006). However,

detailed report on comparative investigation of LS, MS and B5 media is lacking in case of stevia. LS medium contains four times higher thiamine than MS medium and omits pyridoxine, niacin and glycine (Hughes, 1981). Compared to this, the thiamine concentration is much higher (10 mg^{-1}) in B_5 medium (Mehta and Bhatt, 1990). However, Bhaskaran and Jayabalan (2005), reported the superiority of MS medium over B_5 medium for micropropagation of *Eclipta alba* (Asteraceae family).

2.3.5. Optimization of concentration of the medium for multiplication:

Mitra and Pal (2007) reported that the use of full strength MS medium for stevia tissue culture. Osterc *et al.* (2005) reported that ½ strength of MS medium resulted into better multiplication of chestnut nodal explants but increasing the $NH_4NO_3^-$ (medium concentration) resulted in decreased multiplication after certain level and shoot elongation was better on half strength than full strength of MS medium.

In *Laptinella nana* L. (Compositeae family), higher concentration of MS salts was reported to be inhibitory for growth and multiplication (Carson and Leung, 1994). The adverse effects of 1 ½ strength of MS medium may be due to the increased salt concentration upto toxic level especially Cl^- condition was supposed to inhibit NO_3^- uptake (Deane, 1990; Abrol, 1990) whereas, Yashita and Kohne (1990) suggested that higher concentration the other molecules may also get accumulated in plant cells and might be injurious.

2.3.6. Positional effect of explants on mother plant:

Difference in cell division and bud regeneration ability between juvenile and adult plant under *in vitro* conditions has been reported by Stoutemyer and Britt (1965). In case of tree species also, the initial position of isolated explants on its mother plant has been reported to influence its morphogenic expression under *in vitro* conditions (Ramakanthan and Jasrai, 1997). Becerra *et al.* (2004) correlated that the regeneration capacity with aging, as probably did the endogenous content of auxin, which could be associated with the loss of morphogenic capacity. Nhut *et al.,* 2006 reported regeneration from juvenile plant parts of *Gerbera jansanii*.

Contrary to the findings of Rao and Farrook (1997), who reported multiple shoot formation from only nodal cuttings taken from the middle of the young lateral branches, Pilate *et al.* (1989) suggested that mobile hormones synthesized in shoot tip and its young leaves or in the roots and transported downwards (Auxins) or upwards (Cks) in the stem which led to differential cell division and development response in differentially aged explants.

2.3.7. **Effects of different intensities of light on multiplication:**

Fwrnkrage (1990) reported that light intensity has a significant effect on shoot multiplication and *in vitro* growth of plants. In the reports of Karhu and Hokala (1990), medium level of light intensity was found to have no effect on shoot development whereas, Alphonse *et al.* (2002) observed that exposure of *Arthichoke* (Asteracae member) cultures to lower light intensity (20 μmol m^{-2} s^{-1}) was more suitable for shoot elongation and multiplication along with higher number of leaves as compared to those produced under high light intensity (60 μmol m^{-2} s^{-1}). Sevenson (2002) whiles working with *Aristolachia mansheriensis*, found lowest multiplication rate under low light intensity as compared to higher multiplication with medium light

intensity. The inhibition of growth by high light intensity might have the involvement of low IAA oxidase activity (Seibart *et al.,* 1975). According to Puargpaka *et al.* (2001), plant growth is related to the function of growth hormone like auxin, which is sensitive to high light intensity and cytokinins act in concert with auxin in plant tissue culture. However, the higher growth and multiple shoot formation under medium light intensity may not seem to be directly related to the hormonal balance but may be the result of evolution of CO_2 in optimum concentration above the agar (Pierik, 1989). Increased elongation of shoots under low light intensity is reported to be related to increased synthesis of endogenous gibberellins due to gene expression for gibberellin biosynthesis is found to be regulated by light intensity (Sunduwhar, 2006).

2.3.8. Effect of photoperiod on multiplication and development of stevia shoots:

Ferreira and Handro (1987) concluded that the stevia bud initiation was better in the light, in medium with BA(2.0 mgl^{-1}) while in the dark, shoots elongated faster.

Bhatia and Ashwath (2005) reported that the light acts as main regulator of the ultra structural organization of plant photosynthetic apparatus. Sudarson *et . al*.(2003) suggested that in the absence of light proper chlorophyll development in plants might have hampered and therefore etiolated shootlets were produced. Reduced multiplication and development could have related to the release of methane and ethylene due to dark condition. Light stimulated synthesis of enzymes and increased metabolic activities sometimes compensated by reverse processes during dark periods (Sawhnay and Naik, 1990).

Xu *et. al*.(1997) reported stem elongation under short day conditions in rosette plants which was mediated by GA_3. Several reports established the increased

availability of NO_3^- under short light duration (Sopory *et. al.* 1994). Moreover, increased synthesis and accumulation of different proteins on exposure to short light period after a long dark period or vise-versa was reported by Mullet (1988).

Stiles and Cocking (1969) reported longer exposure periods than the critical limit of light may interfere to endogenous hormonal balance.

2.3.9. Effect of number of subcultures / multiplication cycles on multiplication:

How and Keng (2003) obtained satisfactory number of shoots on medium supplemented with 0.5 mgl^{-1} BA. Tiwari *et al.* (2001) also reported the increase in multiplication rate with the number of subculture passage. Samanthi *et al.* (2004) reported that single exposure to low concentration of BA reprogrammed the development throughout the shoot apex whereas, Sreedavy *et al.* (2002) reported gradual decrease of ribotide during subculturing passage.

2.3.10. Influence of different sugars on multiplication of shoots:

Cuenoa and Vietez (2000) reported better growth and development of different spp. on maltose or fructose augmented media than in Sucrose. Baskaran and Jayabalan (2005) reported superiority of sucrose as carbon source. Sucrose has been preferred as the carbon source in tissue culture of stevia (Bondarev *et. al.* 2003) while fructose was used occasionally for plant tissue culture (Silva *et al.,* 2005). Pritchard *et. al* . (1991) explained that the capacity of the *in vitro* plants to absorb, metabolize, assimilate and utilize the carbon at a low concentration depends upon change in osmotic potential of the media. However, effect of maltose for greening plants in tissue culture has been explained as an effect of medium osmolarity (Zhou *et al.,*

1991) whereas the poor response on lactose supplemented media may be related to lower activity of the enzyme β- galactosidase (Fowler *et. al.,* 1982). Amtrosio and De melo (2004) found that the carbohydrates supplemented to the plant tissue during the previous culture phases got accumulated sometimes in large amount.

2.3.11. Optimization of explant inoculation density:

Adelberg (2004) observed that the 5.5 ml of media per explant was optimum for multiplication of *Alocasia microrahiza* on liquid media whereas, on semisolid media, highest multiplication was obtained on somewhat higher ratio (6.06 ml /explant) of inoculation density. Hohe *et al.* (2001) reported changed growth pattern under changed aeration with air instead of CO_2 enriched air whereas, Eun Joo and Youup (2005) obtained highest numbers of shoots were when higher amount (12.5 ml per explant) of media per explant was used.

2.4. Rooting stage:

2.4.1. Effect of nutrient medium concentration on rooting:

Yang and Chang (1979) observed satisfactory rooting on full strength MS nutrient medium. However, it was also observed that the transfer of rooted shoots from high strength medium to less concentrated one enhanced rooting (Filho *et al.,* 1992; Jaime and Da selva, 2004). Bondarev *et al.* (2003) found better elongation of root on 1/3 length of MS medium. However, rooting of shoots was also observed MS medium devoid of growth regulators (Ferreira and Handro, 1988). Same workers also reported better root branching in stevia by supplementation of LS medium with IBA (1.0 mgl^{-1}).

2.4.2. Effect of different PGRs on rooting:

Ferreira and Handro (1988) obtained shorter roots on auxins supplemented medium and reported that the 4 to 6 cm long shoots produced better roots.

Sawanson *et al.* (1992) reported that 1.0 mgl^{-1} NAA being the best supplement on media for rooting in stevia whereas, Tamura *et al.* (1984) observed better rooting on MS medium supplemented with 0.5 mgl^{-1} IBA. Contradictory to this, Fotopoulus and Sotiropoulos (2007) who observed increase in mean root number by increasing auxin concentration whereas, NAA and IAA at higher concentration (2.0 mgl^{-1}) proved better for root initiation in Gladiolus cv. Peach Blossom (Priyakumari and Sheela, 2005).

Constantine ovici and Cachita (1997) and Yang and Chang (1979) reported occurrence of callus formation on treatments containing PGRs. Pushpita *et al.* (1997) reported that the several factors influence the rooting process including auxin which may affect uptake of macronutrients and water uptake from the culture medium. The previous culture supplementations and conditions were also found to affect the rooting process as observed by Morini *et al.* (2003).

Ault (2002) found IBA as an effective PGR for the rooting in various members of Asteraceae also. However, IBA found to rapidly metabolized to IBA aspartate and other conjugate with peptides action (Salisbury and Ross, 1996) which also affected by endogenous level of auxins (Chandra *et al.,* 1999). The inhibition of root initiation and root elongation by higher concentration (2.0 mgl^{-1}) of auxin was reported to be correlated with ethylene production (Klee and Estelle, 1991).

Golaz and Pilet (1995) reported production growth inhibitors by root tip/cap in the presence of light. Other factors viz. amount of PGRs supplemented, their polar

transport and accumulation was also reported to affect rooting (Taiz and Zaiger, 2002).

2.4.3. Effect of combination of auxin on rooting:

Saravanan and Nadarajan (2005) observed the least response of shoots when 0.5 mg^{-1} IAA was used for rooting in *Gloriosa superba* L.. However, better effect of the combination of IBA and IAA was reported by Hassan and Roy (2006).

2.4.4. Effect of number of nodes on rooting:

Ferreira and Handro (1988) stated that the rooting process is affected by the presence of nodes; whereas, Pal *et al.*(2005) reported better growth and development of roots from shoots having more number of nodes as compared to shoots with single or low number of nodes. However Biren and Halevy (2005) stated about the promotion of rooting by enhanced cambial activities in higher plants. Tadhani *et al,* (2006) also observed better rooting response in longer stevia shoots (4 to 6 cm) having atleast 3 to 4 nodes.

2.4.5. Effect of activated charcoal on *in vitro* rooting:

Maynard and Bassak (1987) correlated the root elongation process and the faster metabolism of endogenous auxins in the dark surroundings. Winkle and Pullman (1995) summarized the beneficial effect of AC which include adsorption of components of medium, agar impurities, and inhibitory growth byproducts, media darkening, pH stabilization and catalyzed breakdown of sucrose during sterilization with subsequent absorption of hydroxyl methyl furfural.

2.5. Acclimatization stage:

Constantine ovici and Cachita (1997) observed that the rooting was essential before plantlets were transferred to *ex vitro* condition. Sivaram *et al*. (2003) achieved 70 % survival rate of *Stevia rebaudiana* plantlets at the hardening phase on cocopeat substrate. However, Morini *et al*. (2003) encountered severe problems in Stevia plant acclimatization with highest survival value not exceeding 80% due to mould attack and rapid dehydration. Tadhani *et al*. (2006) who successfully hardened regenerated *S. rebaudiana* plantlets on soil: sand s(1:1) with 70 percent survival rate and suggested treatment of *in vitro* raised stevia plants with 0.1 % bavistin for 2 min. to avoid fungal attack.

MATERIALS AND METHODS

The present investigation on **"***In vitro* propagation of stevia (*Stevia rebaudiana* Bertoni)" was conducted at Department of Agricultural Botany, Bansilal Amritlal College of Agriculture, Anand Agricultural University, Anand during 2004 - 2007.

The general, procedure for the initiation and maintenance of cultures and different analytical methods in this context are embodied in this chapter. Moreover, a brief description of methods as well as chemicals, plastic items and glasswares used is provided here.

3.1. Experimental Material

The experimental material comprised of five months old elite plants of *stevia rebaudiana* Bertoni grown in botanical garden of Department of Agricultural Botany, Bansilal Amrutlal College of Agriculture, Anand Agricultural University, Anand, for initiating axenic cultures. The subsequent *in vitro* studies were done using aseptically grown material.

3. 2. Culture Media

3.2.1 Chemicals

The chemicals used were of superior extra pure grade and were obtained from different reputed companies *viz.* Qualigens, Loba Chemi, SRL, E.Merck and Sigma Chemicals while, the soil substitutes required during hardening *viz.* cocopeat and vermiculite etc. were obtained from the local market.

3.2.2. Culture vessels and plastic items

Depending upon the type of culture, Borosilicate test tubes (150x25mm and 150 x 50mm and 125x50mm size), 'Kasablanka' make round bottles (size 300 ml) and

conical flasks (Borosil 150-250 ml) were used while the plastic items were obtained from 'Tarson'. Before use, all glasswares and plastic items were cleaned with labolene (E. Merck) followed by rinsing under clean running tap water. Finally, all the glass wares were rinsed with single distilled or grade '2' Millipore purified water and oven dried at 60^{o} C for 3 hours.

3.2.3. Preparation of Media

In the present study Gamborg *et al,* (1965), Linsmair and Skoog (1969), Murashige and Skoog (1992), Nitsch and Nitsch (1969) and White's (1963) media were tried as basal for different experiments. Composition of different media used is given in Table No. 1 under appendix 1

Separate stock solutions were prepared according to the composition of medium and were stored at 5º C. Culture media were prepared using their stock solutions made up with Millipore purified grade '1' water, the pH of the media was adjusted to 5.7 ± 0.1 using either 0.1 N. sodium hydroxide or 0.1 N hydrochloric acid.

3.2.4. Sterilization of media and culture vessels

Different volume of boiled media after thoroughly dissolving agar (0.9%) were dispensed in to culture vessels depending upon the experimental requirement.

After closing with polypropylene caps or cotton plugs and aluminum foils, the vessels containing media were sterilized by autoclaving at a pressure of 1.05 kg/ cm^2 and 121º C for 20 minutes using a vertical autoclave. Petriplates and tools like scalpels, cork borer spatula, forceps etc. were sterilized at the same conditions for 30 minutes.

The heat labile compounds like hormones were filter sterilized instead of autoclaving by filtering their stock solution through 0.22 filters fixed in a previously autoclaved filter sterilization unit (Tarson) in to a sterile container under suction pressure. The filtered solutions were then aseptically added to the autoclaved culture medium in required volume.

3.2.5. Aseptic conditions

The various operations related to tissue culture *viz.* surface sterilization, inoculation, and sub culturing etc. were carried out in sterile airflow under a laminar airflow hood (Microfilt, India). The culture vessels were exposed to U.V. light for 20 minutes to get the vessels sterilized. The tools used for inoculation and sub culturing were frequently flame sterilized and subsequently placed in to glass bead sterilizer (Dent-eq) during different inoculation and subculturing operations.

3.2.6. Preparation of explant and surface sterilization

Different plant parts *viz.,* nodes or shoot tip of 1- 2 cm size (according to experimental requirement) were selected and collected from healthy field grown plants.

These explants were thoroughly washed first with tap water followed by washing with sterilized grade '1' water containing few drops of the surfactant 'Tween 20' solution. Explants were surface sterilized using proper surface sterilant for appropriate duration and finally washed three times with sterile water to remove the traces of sterilization agent.

3.3. Culture Techniques

3.3.1. Inoculation of explants

Suitable starting material/explants after surface sterilization treatment were placed on appropriate medium by maintaining the polarity of the explant.

3.3.2. Subculture techniques

Subculturing were done at regular intervals of 21 to 45 days in accordance of physiological stage of plant material and the requirement of the experiment.

3.4. Culture Conditions

All the cultures have been incubated in a culture room maintained at 25 ± 2ºC, 40 to 60% relative humidity and 16/8 hrs light/dark regime, except for cases where specific physiological conditions were required. The liquid media based cultures were kept on a gyrotary shaker kept at 95 rpm under the same culture conditions.

3.5. Hardening

The *in vitro* rooted shoots were carefully taken out from the culture vessels and gently washed with sterile water so as to remove the agar and medium adhered to it. The shoots were then dipped in 1 % aqueous solution of Bavistin for 10 to 15 minutes followed by a wash with tap water.

The plantlets were transferred into 10 x 9 cm plastic cups containing moist, autoclaved substrates and covered by plastic bags, watered daily to maintain high relative humidity. Plantlets were placed initially in a growth room under 16 hrs photoperiod at 25º C. Plantlets were acclimatized over a period of three to four weeks by exposure to room temperature. The humidity was gradually reduced by opening the plastic bags slightly until plants were ready for transfer to the net house. In the net house, plantlets were maintained in pots and watered every 2 days. After 3 to 4 weeks period, the plantlets were transferred to the open field.

3.6. Observations

The changes in morphological characters respective to various physiological / culture stages were observed and recorded during various experiments on daily basis or at intervals of 7 days, 14 days and 21 days. The various parameters recorded at different stages are presented under each head as below.

3.6.1. Surface sterilization

A) **Percentage contamination**

Contamination was recorded after seven days of inoculation as the total number of explants contaminated out of total number of explants inoculated and expressed in percentage.

B) **Percentage of survived material**

For percentage of survived material, the numbers of dried explants were recorded and remaining explants were expressed in percentage against the total number of explants inoculated.

3.6.2. Culture establishment

A) Percentage of response

The total number of shoot bud sprouted per explant were recorded and expressed against the total number of nodes present.

B) Days to initiation of sprouting

The total number of days taken by the axillary buds for sprouting was counted from the day of inoculation and was considered as days to initiation of sprouting.

3.6.3. Multiplication stage

A) Multiplication response/rate

Multiplication rate was calculated by dividing the total number of shoots by initial number of shoots from an explant inoculated.

B) Length of shoot

Length of shoots was measured from the base to tip and finally the average of the measurements was considered for expressing the length of shoot in response to the particular medium. The formula used for average shoot length was:

$$\frac{L_1 + L_2 + L_3 \text{----------} Lx}{\text{Total number of shoots present}}$$

Where, L1 L2 L3…….Lx were length of different shoots.

C) Shoots per explant

Number of shoots measuring more than 1 cm in length was counted per explant inoculated.

D) Number of nodes per shoot.

The total number of nodes observed was counted against total number of shoots present.

3.6.4. Rooting stage

A) Days to root initiation:

The total number of days taken by the explants to initiate roots was counted against total number of shoots present

B) Number of roots per shoot:

The total number of roots of about 1 mm length, induced from each shoot was counted.

C) Length of roots:

The length of each root was measured from the base of shoot and the average value of the measurement was scored.

D) Number of non-functional / callus interveined roots:

The total number of roots interveined by the callus at junction of root and shoot were counted against the total number of roots produced.

3.6.5. Acclimatization phase

A) Number of survived plants:

The total number of plantlets taken out from the *in vitro* conditions to *ex vitro* conditions and survived after 21 days from transfer were counted.

3.7. Experimental Procedures

3.7.1. Stage Zero: Mother plant selection

Before surface sterilization, the mother plant selection was done for separating out plant parts which were served as explant. For this purpose five months old elite plants characterized by profuse type of branches at the base and having minimum half a meter height were chosen. Moreover, parts from only vegetative plants were taken and parts from reproductive stage plants were avoided.

3.7.2. Surface sterilization experiment

Explants of stevia were surface sterilized by using six types of sterilization agents and their combinations sequentially, for different durations of exposure during various experiments. The surface sterilization treatments used in this study are:

(A) Efficiency of different antibacterial agents in surface sterilization:

1) Cefotaxime (200 ppm) for 10 min.

2) Kanamycin (200 ppm) for 10 min.

3) Streptomycin (200 ppm) for 10 min.

4) Tetracycline (200 ppm) for 10 min.

(B) Effect of heavy metal in reducing contaminants:

1) 0.01% $HgCl_2$ for 10 min.

2) 0.1% $HgCl_2$ for 10 min.

3) 0.5% $HgCl_2$ for 10 min.

4) 0.01% $HgCl_2$ for 20 min.

5) 0.1% $HgCl_2$ for 20 min.

6) 0.5% $HgCl_2$ for 20 min.

(C) Sequential surface sterilization:

The experiment was conducted to find out the possibilities of the use of alternate sterilants apart from $HgCl_2$ or with minimal use/exposure of $HgCl_2$ to get 100% sterile culture with better health.

1) 200 ppm Streptomycin for 10 min. followed by 0.1% $HgCl_2$ for 10 min.

2) 200 ppm Kanamycin for 10 min. followed by 0.1% $HgCl_2$ for 10 min.

3) 200 ppm Cefotaxime for 10 min. followed by 0.1% $HgCl_2$ for 10 min.

4) 200 ppm Bavistin for 10 min. followed by 0.1% $HgCl_2$ for 10 min.

5) 200 ppm Bavistin for 10 min. followed by 200 ppm Cefotaxime for 10 min.

6) 200 ppm Kanamycin for 05 min. followed by 200ppm Bavistin for 10 min. and lastly 0.1% $HgCl_2$ for 5 min.

3.7.3. Stage one: Culture establishment

A study on establishment of axenic cultures of stevia was done as the first stage for *in vitro* multiplication and for this purpose two experiments were conducted initially and which was followed by other experiments. In the first experiment, two different media i.e. MS (1962) and B_5 (1965) were tried at various strength, while in the second experiment, the best medium found in the first experiment was tried with supplementation of different levels of various growth regulators to find out their effect on establishment. The details of the treatments under different experiments are as follows:

1) Effect of different basal media on establishment and development:

Following treatments consisting of various strength MS and B_5 media along with control were tested to evaluate their effects on culture establishment and initiation:

1) Half strength Murashige and Skoog (1962) medium.
2) Half strength B_5 (Gamborg 1995) medium
3) Full strength Murashige and Skoog (1962) medium.
4) Full strength B_5 medium.
5) Control

2) Effect of auxin: cytokinin (combination) on Establishment

1) MS + 0.05 $mg1^{-1}$ NAA + 0.0 $mg1^{-1}$ BA + 3% S
2) MS + 0.1 $mg1^{-1}$ NAA + 0.0 $mg1^{-1}$ BA + 3% S
3) MS + 0.2 $mg1^{-1}$ NAA + 0.0 $mg1^{-1}$ BA + 3% S

4) MS + 0.4 $mg1^{-1}$ NAA + 0.0 $mg1^{-1}$ BA + 3% S

5) MS + 0.05 $mg1^{-1}$ NAA + 0.05 $mg1^{-1}$ BA + 3% S

6) MS + 0.1 $mg1^{-1}$ NAA + 0.05 $mg1^{-1}$ BA + 3% S

7) MS + 0.2 $mg1^{-1}$ NAA + 0.05 $mg1^{-1}$ BA + 3% S

8) MS + 0.4 $mg1^{-1}$ NAA + 0.05 $mg1^{-1}$ BA + 3% S

9) MS + 0.05 $mg1^{-1}$ NAA + 0.1 $mg1^{-1}$ BA + 3% S

10) MS + 0.1 $mg1^{-1}$ NAA + 0.1 $mg1^{-1}$ BA + 3% S

11) MS + 0.2 $mg1^{-1}$ NAA + 0.1 $mg1^{-1}$ BA + 3% S

12) MS + 0.4 $mg1^{-1}$ NAA + 0.1 $mg1^{-1}$ BA + 3% S

13) MS + 0.05 $mg1^{-1}$ NAA + 0.2 $mg1^{-1}$ BA + 3% S

14) MS + 0.10 $mg1^{-1}$ NAA + 0.2 $mg1^{-1}$ BA + 3% S

15) MS + 0.2 $mg1^{-1}$ NAA + 0.2 $mg1^{-1}$ BA + 3% S

16) MS + 0.4 $mg1^{-1}$ NAA + 0.2 $mg1^{-1}$ BA + 3% S

17) MS + 0.05 $mg1^{-1}$ NAA + 0.4 $mg1^{-1}$ BA + 3% S

18) MS + 0.1 $mg1^{-1}$ NAA + 0.4 $mg1^{-1}$ BA + 3% S

20) MS + 0.2 $mg1^{-1}$ NAA + 0.4 $mg1^{-1}$ BA + 3% S

21) MS + 0.4 $mg1^{-1}$ NAA + 0.4 $mg1^{-1}$ BA + 3% S

22) MS + 3% S.

3.7.4. Effect of light and dark periods on establishment

Various light and dark cycles were tested and analyzed for their effect on bud sprouting and better establishment of aseptic cultures. The treatment details are as follows:

T1) 2 days dark followed by 12 days continuous light.

T2) 4 days complete dark followed by 10 days total light.

T3) 7 days complete dark followed by 7 days complete light.

T4) 2 days complete light followed by 12 days complete dark.

T5) 4 days complete light followed by 10 days complete dark.

T6) 4 days complete light followed by 7 days complete dark followed by 3 days light

T7) 4 days complete dark regime followed by 3 days light regime again
4 days dark regime followed by 3 days dark regime.

T8) 4 days complete light regime followed by 3days dark regime again 4 days light regime followed by 3 days light regime.

3.8. Stage two: Multiplication

3.8.1. Effect of different cytokinins on formation and development of shoots

After successful establishment of cultures, effect of different cytokinins on multiple shoot formation was examined. The explants were cultured on MS medium supplemented with four cytokinins *viz.* BA, Kinetin, Thidiazuron and Zeatin separately at the following concentrations.

Treatment code	Treatment
T1	MS + 0.1 mgl^{-1} BA + 3% S
T2	MS + 0.5 mgl^{-1} BA + 3% S
T3	MS + 1.0 mgl^{-1} BA + 3% S
T4	MS + 2.0 mgl^{-1} BA + 3% S
T5	MS + 5.0 mgl^{-1} BA + 3%S
T6	MS + 0.1 mgl^{-1} Kn + 3%S
T7	MS + 0.5 mgl^{-1} Kn + 3%S
T8	MS + 1.0 mgl^{-1} Kn + 3%S
T9	MS + 2.0 mgl^{-1} Kn + 3%S
T10	MS + 5.0 mgl^{-1} Kn + 3%S
T11	MS + 0.1 mgl^{-1} BA + 3%S
T12	MS + 0.5 mgl^{-1} TDZ + 3%S
T13	MS + 1.0 mgl^{-1} TDZ + 3%S
T14	MS + 2.0 mgl^{-1} TDZ + 3%S
T15	MS + 5.0 mgl^{-1} TDZ + 3%S
T16	MS + 0.1 mgl^{-1} Zn + 3%S
T17	MS + 0.5 mgl^{-1} Zn + 3%S
T18	MS + 1.0 mgl^{-1} Zn + 3%S
T19	MS + 2.0 mgl^{-1} Zn + 3%S
T20	MS + 5.0 mgl^{-1} Zn + 3%S
C	Control

3.8.2. Effect of combination of cytokinins on multiplication

Various combinations of BA and Kn were tested to increase the number of multiple shoots and subsequent development on previously identified culture conditions. The treatments tried are:

Treatment code	PGR combination
T1	MS + 0.1 $mg1^{-1}$ BA + 0.5 $mg1^{-1}$ Kn +3% S
T2	MS + 1.0 $mg1^{-1}$ BA + 1.0$mg1^{-1}$ Kn +3% S
T3	MS + 2.0 $mg1^{-1}$ BA + 2.0$mg1^{-1}$ Kn +3% S
T4	MS + 4.0 $mg1^{-1}$ BA + 0.5 $mg1^{-1}$ Kn + 3% S
T5	MS + 0.1 $mg1^{-1}$ BA + 1.0 $mg1^{-1}$ Kn + 3% S
T6	MS + 1.0 $mg1^{-1}$ BA + 2.0 $mg1^{-1}$ Kn + 3% S
T7	MS + 2.0 $mg1^{-1}$ BA + 0.5 $mg1^{-1}$ Kn + 3% S
T8	MS + 4.0 $mg1^{-1}$ BA + 1.0 $mg1^{-1}$ Kn + 3% S
T9	MS + 0.1 $mg1^{-1}$ BA + 2.0 $mg1^{-1}$ Kn + 3% S
T10	MS + 1.0 $mg1^{-1}$ BA + 0.5 $mg1^{-1}$ Kn + 3% S
T11	MS + 2.0 $mg1^{-1}$ BA + 1.0 $mg1^{-1}$ Kn + 3% S
T12	MS + 4.0 $mg1^{-1}$ BA + 2.0 $mg1^{-1}$ Kn + 3% S

3.8.3. Effect of different media on multiplication and development

Five media MS, B_5, LS, Nitsch and Nitsch and White's at full strength were evaluated for their effects on multiplication of shoots. All the basal media were fortified with 3% sucrose solidified with 0.9% (w/v) Agar and supplemented with 0.5 $mg1^{-1}$ BA. the details of the media used is presented in Table No. 1 (Appendix 1). Treatment wise description is as follows:

Treatments	Media
1)	Gamborg's (B_5) medium
2)	Linsmair and Skoog medium
3)	Murashige and Skoog medium
4)	Nitsch and Nitsch medium
5)	White's medium

3.8.4. Selection of best medium for multiple shoot formation

As in the first experiment, three media *viz.* B5, MS and LS showed comparable results as compared to Nitsch or White's media. Therefore these media were tried at half and full strength to examine any better effects.

Treatment	Media
T1)	Full strength Gamborg's B_5 medium
T2)	Half strength B_5 medium
T3)	Full strength Linsmair and Skoog media
T4)	Half strength Linsmair and Skoog medium
T5)	Full strength Murashige and Skoog medium
T6)	Half strength Murashige and Skoog medium

3.8. 5. Optimization of concentration of the medium for multiplication:

Based on the results of previous experiments an experiment was carried out to optimize the concentration of MS medium for further increase in multiplication of shoots. For this purpose half strength, 3/4 strength, 1/1 (full) strength and 1 ½ (1.5) strength of MS medium were examined.

3.8.6. Positional effect of explants on mother plant:

Influence of the position of the explant on the mother plant before incision on its morphogenic expression under *in vitro* conditions was studied using the nodes as under:

T1 = Uppermost node T2 = Subapical node T3 = Mediary node

T4 = Second lower node T5 = Lowermost or Basal node

3.8.7. Effect of number of nodes on multiplication:

To find out the effect of number of nodes per explant on shoot formation and multiplication, a comparison was made with five explants differed in the number of nodes *viz.* single node, shoot with 2 nodes, shoot with 3 nodes, shoot with 4 nodes and shoot with 5 nodes.

3.8.8. Effects of different intensities of light on multiplication

Trials were conducted to evaluate the performance of *in vitro* grown shoots containing two under three different light intensity regimes including low light intensity

(20 μmol m^{-2} s^{-1}), medium light intensity (40 μmol m^{-2} s^{-1}) and high light intensity (60 μmol m^{-2} s^{-1}).

3.8.9. Effect of different photoperiods on multiplication and development of stevia shoots:

An experiment was carried out to find out photomorphogenic effects of various light/dark period regimes. For this purpose, nodal explants (1 cm size) were inoculated in vessel containing MS medium supplemented with 0.5 $mg1^{-1}$ BA and 3% sucrose. The cultures were incubated at 25 ± 1 ºC temperature regime and grown under the following photoperiods:

Treatment	Photoperiod
T1)	0 hrs light / 24 hrs dark
T2)	8 hrs light / 16 hrs dark
T3)	12 hrs light / 12 hrs dark
T4)	16 hrs light / 8 hrs dark
T5)	24 hrs light / 0 hrs dark

3.8.10. Influence of different sugars on multiplication of shoots:

Nodal segments were cultured on MS medium supplemented with 0.5 $mg1^{-1}$ BA and five types of sugars *viz.* Fructose, Glucose, Lactose, Maltose and Sucrose at various concentrations (1 % to 5 %) to evaluate their influence on shoot multiplication. The treatment wise detail is as follows.

Treatment No.	Treatment (sugar)
T1)	MS + 0.5 $mg1^{-1}$ BA + 1% Fructose
T2)	MS + 0.5 $mg1^{-1}$ BA + 1% Glucose
T3)	MS + 0.5 $mg1^{-1}$ BA + 1% Lactose
T4)	MS + 0.5 $mg1^{-1}$ BA + 1% Maltose
T5)	MS + 0.5 $mg1^{-1}$ BA + 1% Sucrose
T6)	MS + 0.5 $mg1^{-1}$ BA + 2% Fructose
T7)	MS + 0.5 $mg1^{-1}$ BA + 2% Glucose
T8)	MS + 0.5 $mg1^{-1}$ BA + 2% Lactose
T9)	MS + 0.5 $mg1^{-1}$ BA + 2% Maltose
T10)	MS + 0.5 $mg1^{-1}$ BA + 2% Sucrose
T11)	MS + 0.5 $mg1^{-1}$ BA + 3% Fructose
T12)	MS + 0.5 $mg1^{-1}$ BA + 3% Glucose
T13)	MS + 0.5 $mg1^{-1}$ BA + 3% Lactose

T14)	MS + 0.5 $mg1^{-1}$ BA + 3% Maltose
T15)	MS + 0.5 $mg1^{-1}$ BA + 3% Sucrose
T16)	MS + 0.5 $mg1^{-1}$ BA + 4% Fructose
T17)	MS + 0.5 $mg1^{-1}$ BA + 4% Glucose
T18)	MS + 0.5 $mg1^{-1}$ BA + 4% Lactose
T19)	MS + 0.5 $mg1^{-1}$ BA + 4% Maltose
T20)	MS + 0.5 $mg1^{-1}$ BA + 4% Sucrose
T21)	MS + 0.5 $mg1^{-1}$ BA + 5% Fructose
T22)	MS + 0.5 $mg1^{-1}$ BA + 5% Glucose
T23)	MS + 0.5 $mg1^{-1}$ BA + 5% Lactose
T24)	MS + 0.5 $mg1^{-1}$ BA + 5% Maltose
T25)	MS + 0.5 $mg1^{-1}$ BA + 5% Sucrose
T26)	MS + 0.5 $mg1^{-1}$ BA + No sugar

3.8.11. Optimization of inoculation density of single node explant:

To determine optimal inoculation density of single node explants on semisolid media, different number of single nodes (5, 10, 20 and 30) were inoculated on 40 ml of media as follows:

Treatments	**Inoculation density**
T1	5 explants/40 ml medium
T2	10 explants/40 ml medium
T3	20 explants/40 ml medium
T4	30 explants/40 ml medium

3.8.12. Effect of number of subculture on multiplication:

Effect of the number of subcultures on multiplication was evaluated by recording multiplication number upto 8th multiplication cycle. Nodal explants were inoculated on full strength MS medium supplemented with 0.5 mgl^{-1} BA and 3 % sucrose. Cultures were maintained under 40 µmol m^{-2} s^{-1} light intensity for 8 hrs. of photoperiod. Periodical subculturings were made every 21st day after inoculation and observations were recorded before each subculture.

3.9.1. Stage three: Rooting phase

3.9.2. Effect of the concentration of nutrient medium on rooting:

Various concentrations of MS medium (1/4, 1/3, 1/2 and full strength) were used to investigate their effect on rooting of stevia shoots.

3.9.3. Effect of different PGRs on rooting:

Different concentrations and types of auxin (IAA, IBA and NAA) were used at different concentrations (0.5 mgl^{-1}, 1.0 mgl^{-1} and 2.0 mgl^{-1}) for studying its effect on rooting:

Treatment code	Medium and supplementation
T1	1/2 MS + No auxin + 2 % S
T2	1/2 MS + 0.5 mgl^{-1} IAA + 2 % S
T3	1/2 MS + 0.5 mgl^{-1} IBA + 2 % S
T4	1/2 MS + 0.5 mgl^{-1} NAA + 2 % S
T5	1/2 MS + 1.0 mgl^{-1} IAA + 2 % S
T6	1/2 MS + 1.0 mgl^{-1} IBA + 2 % S
T7	1/2 MS + 1.0 mgl^{-1} NAA + 2 % S
T8	1/2 MS + 2.0 mgl^{-1} IAA + 2 % S
T9	1/2 MS + 2.0 mgl^{-1} IBA + 2 % S
T10	1/2 MS + 2.0 mgl^{-1} NAA + 2 % S

3.9.4. Effect of the combination of auxins on rooting:

Further trials were conducted to find out the possibilities for improvement in rooting of shoots by incorporating three different combinations of two auxins (i) 0.5 mgl^{-1} IAA + 0.5 mgl^{-1} IBA (ii) 0.5 mgl^{-1} IAA + 0.5 mgl^{-1} NAA and (iii) 0.5 mgl^{-1} IBA + 0.5 mgl^{-1} NAA using MS medium along with (iv) control consisting of previously identified best PGR (1mgl^{-1} IAA) .

3.9.5. Effect of activated charcoal on *in vitro* rooting:

An experiment was conducted with eight treatments containing different auxin along with / without activated charcoal to improve the rooting response of stevia shoots and to compare their effect on rooting. The details of the treatments are as follows:

Treatment Code	Treatments
T1	Control 1/2 MS + 2% S
T2	1/2 MS + 0.4 gl^{-1} AC + 2% S
T3	1/2 MS + 0.5 mgl^{-1} IBA + 2% S
T4	1/2 MS + 0.5 mgl^{-1} IBA + 0.4 gl^{-1} AC + 2% S
T5	1/2 MS + 0.5 mgl^{-1} IAA + 2% S
T6	1/2 MS + 0.5 mgl^{-1} IAA + 0.4 gl^{-1} AC + 2% S
T7	1/2 MS + 0.5 mgl^{-1} NAA + 2% S
T8	1/2 MS + 0.5 mgl^{-1} NAA + 0.4 gl^{-1} AC + 2% S

3.9.6. Effect of number of nodes on rooting:

Shoots having different number of nodes (1 node, 2 nodes, 3 nodes, 4 nodes, 5 nodes and 6 nodes) were inoculated on root induction medium to evaluate their effect on root growth and development

4.10. Stage four: Hardening phase

4.10.1. Effect of different potting mixtures on survival of plantlets:

Different substrates *viz.* cocopeat, farm yard manure (FYM), sand, soil and vermiculite were tried alone or in combinations as potting mixture to evaluate their effect on survival of plantlets *ex vitro*. The treatments tried are as follows:

Treatment code	Potting mixture
T1	Cocopeat alone
T2	Farm yard manure (FYM) alone
T3	Sand alone
T4	Soil alone
T5	Vermiculite alone
T6	Cocopeat: Sand (1:1)
T7	Cocopeat: Soil (1:1)
T8	Cocopeat: FYM (1:1:)
T9	Cocopeat :Vermiculite (1:1)
T10	FYM :Sand (1:1)
T11	FYM: Soil (1:1)
T12	FYM :Vermiculite (1:1)
T13	Sand: Soil (1:1)
T14	Sand: Vermiculite (1:1)
T15	Soil: Vermiculite (1:1)
T16	Cocopeat :FYM: Sand (1:1:1)
T17	Cocopeat :FYM: Vermiculite (1:1:1)
T18	Cocopeat : Soil : Vermiculite (1:1:1)
T19	Cocopeat : Sand: Soil (1:1:1)
T20	FYM : Sand : Soil (1:1:1)
T21	Sand : Soil: Vermiculite (1:1:1)
T22	Sand: Soil : FYM : Vermiculite (1:1:1:1)
T23	Cocopeat :FYM : Sand : Soil (1:1:1:1)
T24	Cocopeat : Sand : Soil : Vermiculite (1:1:1:1)
T25	Cocopeat: FYM : Sand: Vermiculite (1:1:1:1)
T26	Cocopeat: FYM : Sand : Soil: Vermiculite (1:1:1:1:1)

3.10.2. Field planting:

Properly hardened plantlets (45 days old) were transplanted in the field in large numbers and studied for its field survival on sandy loom (Goralu) soil at the Botanical Garden of the Department.

3.11. Statistical analysis:

The data pertaining to various characters will be statistically analyzed by the method described Sneder and Cohran (1963) i.e. completely randomized design.

RESULTS AND DISCUSSION

4.1. Surface sterilization experiments:

4.1.1. Surface sterilization by $HgCl_2$

Various concentrations of $HgCl_2$ *viz.* 0.01 % to 0.5 % for 10 min. and 20 min. were tried to investigate its effectiveness in controlling surface contamination and improved health of explants. The observations are presented in Fig.:1.

Among different treatments tried, none was found significantly superior to get reasonably healthy and satisfactorily contamination free cultures. The results showed that the least contamination (19.96 %) was obtained when explants were treated with 0.5 % $HgCl_2$ for 20 min. However, the survival percentage of explants was found to be poor (10 %) whereas, the best survival percentage was higher (94 %) when 0.01 % $HgCl_2$ treatment was given to explant for 20 minutes. These results are contradictory to the reports of Tadhani *et al.* (2006), wherein 0.1 % $HgCl_2$ treatment was given for shorter duration of 2 min. after pretreatment with 70 % alcohol. However, use of $HgCl_2$ for longer sterilization duration of > 5 minutes have been mentioned in several reports (Miyagawa *et al.*, 1986; Bondarev *et al.* , 2003; Mitra and Pal, 2007) Mercuric chloride and its soluble salts are efficient sterilants. It acts through the action on protein sulfhydryl groups and disruption of enzyme functions of the microorganisms (EH and S Rev. 2005). Difference in the exposure time of the explants to surface sterilization agents in the present study may be due to the size difference of the explants as compared to the studies by the other workers.

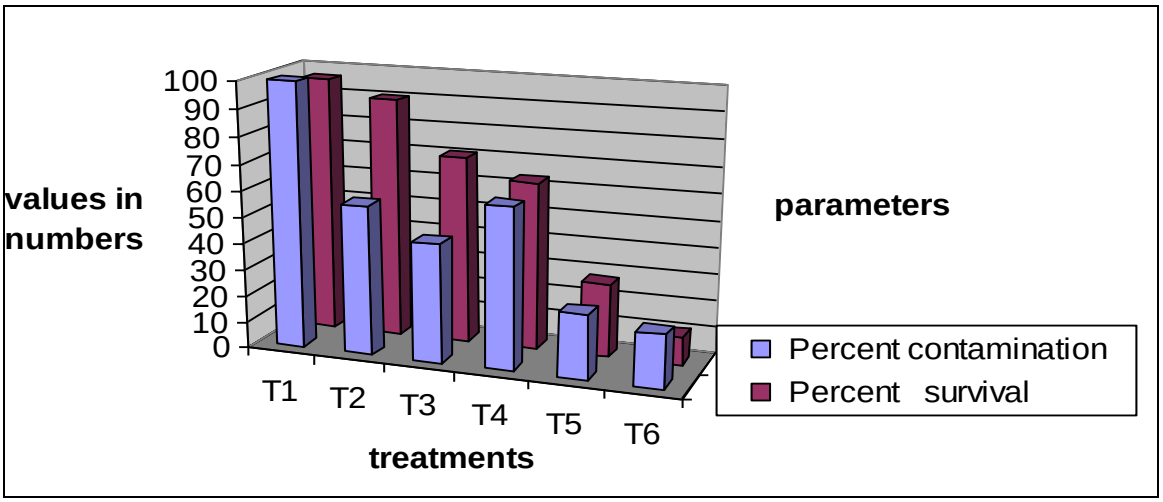


Fig. 1: Effects of surface sterilization by $HgCl_2$ on percentage contamination and survival of explants

4.1.2. Surface sterilization by antibiotics

Four different antibiotics *viz.* kanamycin, streptomycin, cefotaxime and tetracycline were tried as surface sterilant for total elimination of contamination from explant surface in this experiment. The percentage survival was higher (98.8) when kanamycin was used whereas, followed by (94.4) was observed with cefotaxime(Fig. 2) However, all the treatments failed to control fungal contamination. The sudden outbreak of fungal contamination may be related to the collection of explants during monsoon season as reported by Kulkarni (2000).

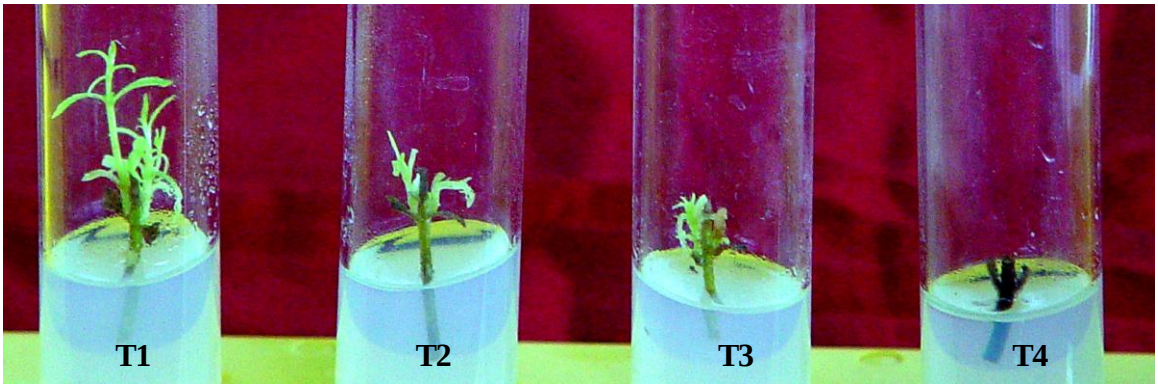


Plate No. 1: Effect of antibacterial agents on explant survival during surface sterilization

4.1.3. Sequential surface sterilization (Application of antibiotics, fungicides and $HgCl_2$):

The experiment was based on six different combinations of antibiotics/ bacteriocides and antifungal agents (3.7.2.C) and were used sequentially to eliminate surface contaminants completely.

Among various treatments tried, treatment No.6 consisting of lower concentration of bavistin (200 ppm) followed by 200 ppm kanamycin and 0.1 % $HgCl_2$ for 5 min. to 10 min. sequentially resulted in complete elimination of surface contaminants and satisfactory survival percentage (90%) of the explants (Fig. 3). Treatment No. 5 consisting of sequential use of 200 ppm Bavistin and 200 ppm Cefotaxime was found least effective (80% contamination) in controlling contamination with 97 % of survival. Better survival of explants were observed when kanamycin and $HgCl_2$ based treatment No.2 was applied. These results are contradictory to the previous reports of stevia surface sterilization wherein single sterilant was found effective in eliminating contaminants which is not true in the present study. However, Kulkarni (2000) reported complete elimination of surface contaminants in *Taxus* Spp. by a combination of antifungal and antibacterial agents along with $HgCl_2$.

Optimum elimination of contaminants (97%) in the present study may be due to very low bioload of microorganisms which might have been gradually decreased during different sequential steps employed for surface sterilization while, moderate survivability of explants might have been due to longer sterilization period (total 20min.).

Irrespective of various reasons, the treatments based on bavistin were found to be less harsh to the explants and showed elimination of fungal contamination (Fig 2) except when the combination of bavistin and cefotaxime was tried (Treat. No. 3). The results are in accordance with that of Mitra and Pal (2007). Bavistin (50 % Carbondezim) is a systemic fungicide belonging to the benzimidazol group. It contains methyl -3-benzimidazol carbamate and acts by interfering with DNA production and post DNA synthesis aspects of fungal cell replication (Singh, 1990).

The less effectiveness of treatment No. 3 may be due to the incompatibility or indiscriminate combinational problems (Phillips *et. al.*, 1981).

Results also showed that pretreatment with kanamycin i.e. Treatment No. 2 resulted in higher survival of explants (Fig. 3). There are several reports of kanamycin as a selective agent and sterilant in tissue culture (Mohammed *et al*., 2005).

Kanamycin comes under the aminoglycosidase group of antibiotics with more specific action on wide variety of metabolic changes including changes in cell permeability, transport, and inhibition of protein synthesis and misreading of the genetic code of bacteria, thus may be less harmful towards plant cells and therefore higher survival.

Based on all the above experiments the best surface sterilization treatment i.e. bavistin (200 ppm) followed by 200 ppm kanamycin and 0.1 % $HgCl_2$ for 5 min. to 10 min. was used to initiate axinic cultures for all future experiments.

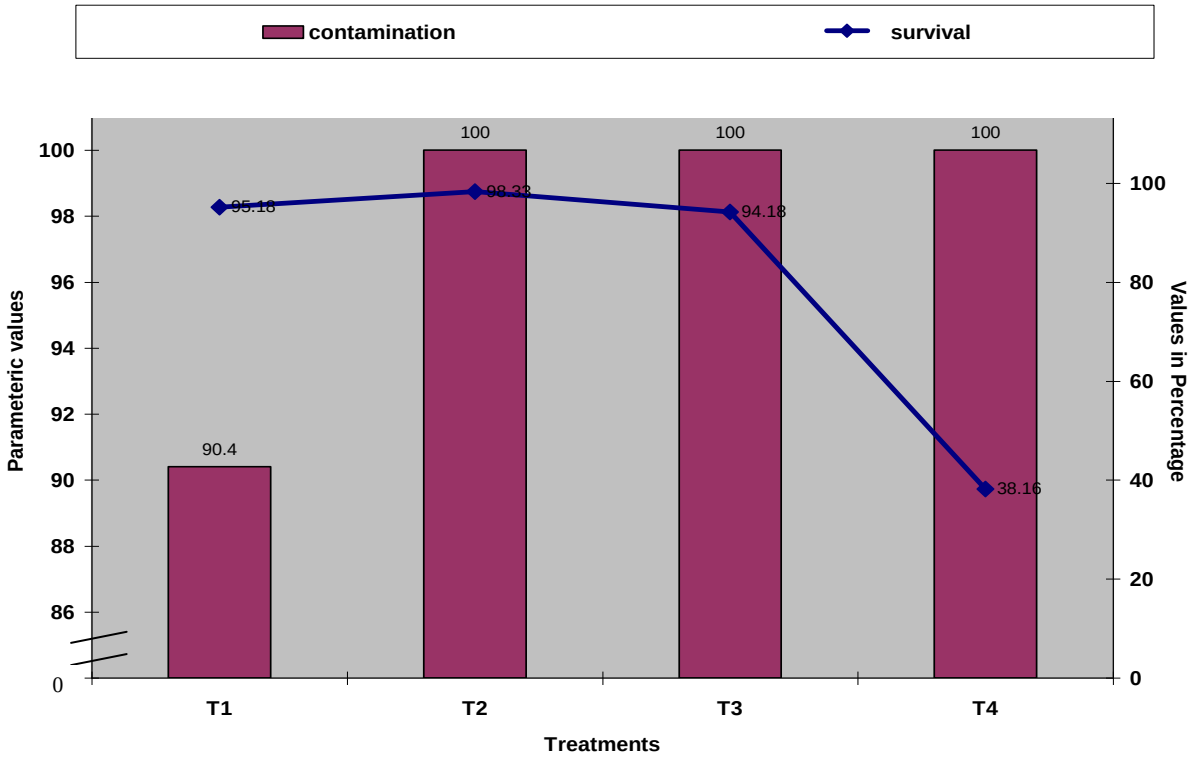


Fig. 2: Surface sterilization by antibiotics

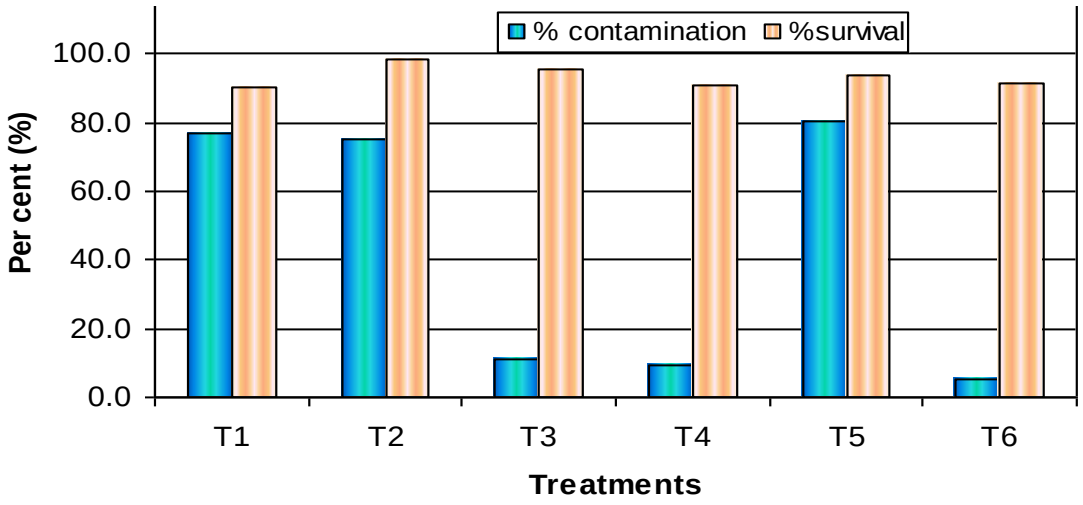


Fig. 3: Effect of sequential application of surface sterilization agents

4.2. Establishment Phase

4.2.1. Effect of different basal media on establishment and growth:

Five treatments including half strength MS medium, full strength MS, half strength B_5 and full strength B_5 medium along with control were tested to evaluate their effects on culture establishment and initiation.

Results showed that the percentage establishment and growth of cultures on full strength MS medium was better (98%) than other media tried while, minimum number of five days were recorded for bud sprouting when full strength B_5 medium was used(Fig. 4). Percentage response of cultures was satisfactory on both MS and B_5 media (12 % and 37 %) when used at full strength whereas decreased growth was observed on half strength B_5 medium which hints at high level of salt requirement for establishment phase.

Miyagawa *et. al.* (1986) reported better establishment of stevia cultures on B_5 media whereas, Nepavim and Vanek (1998) observed better establishment and initiation of cultures on half strength MS medium. However, full strength medium has been proved as a better option for establishment of most of the plants belonging to *Asteraceae* family including stevia (Bondarev *et. al.*, 2003).

Amazaling *et. al.* (1992) observed the promotory effect of nutrient medium on establishment and attributed it mainly to the participation of inorganic ions in regulatory processes and their outcomes. Similarly, the probable reason behind the early sprouting of buds on B_5 medium may be due to the takeover of NO_3^- uptake and assimilation after initial uptake of NH_4^+ leading to an increase in pH and increased uptake of K^+ and SO_4^+ and availability in B_5 medium. Although initial sprouting was fast, further bud development or shoot elongation seems to cease due to the evolution of ethylene (Salisbury and Ross, 2003). Khetrapal *et al.* (1999) reported that the ethylene evolution was more in B5 medium than in MS medium. This may be related to the concentration of total nitrogen in these media (60mM in MS and 27mM in B_5). The less evolution of ethylene could be the reason behind the better overall development on MS medium than B5 medium (Plate 2).

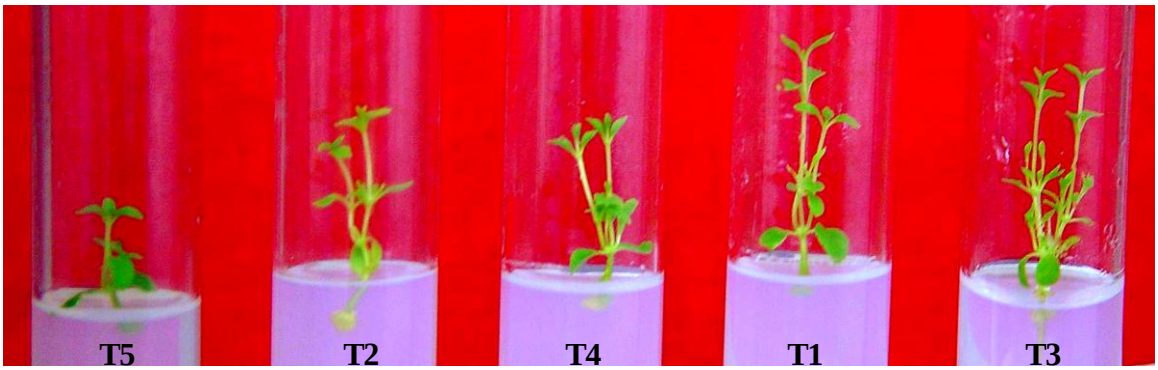


Plate 2: Effect of nutrient media on establishment

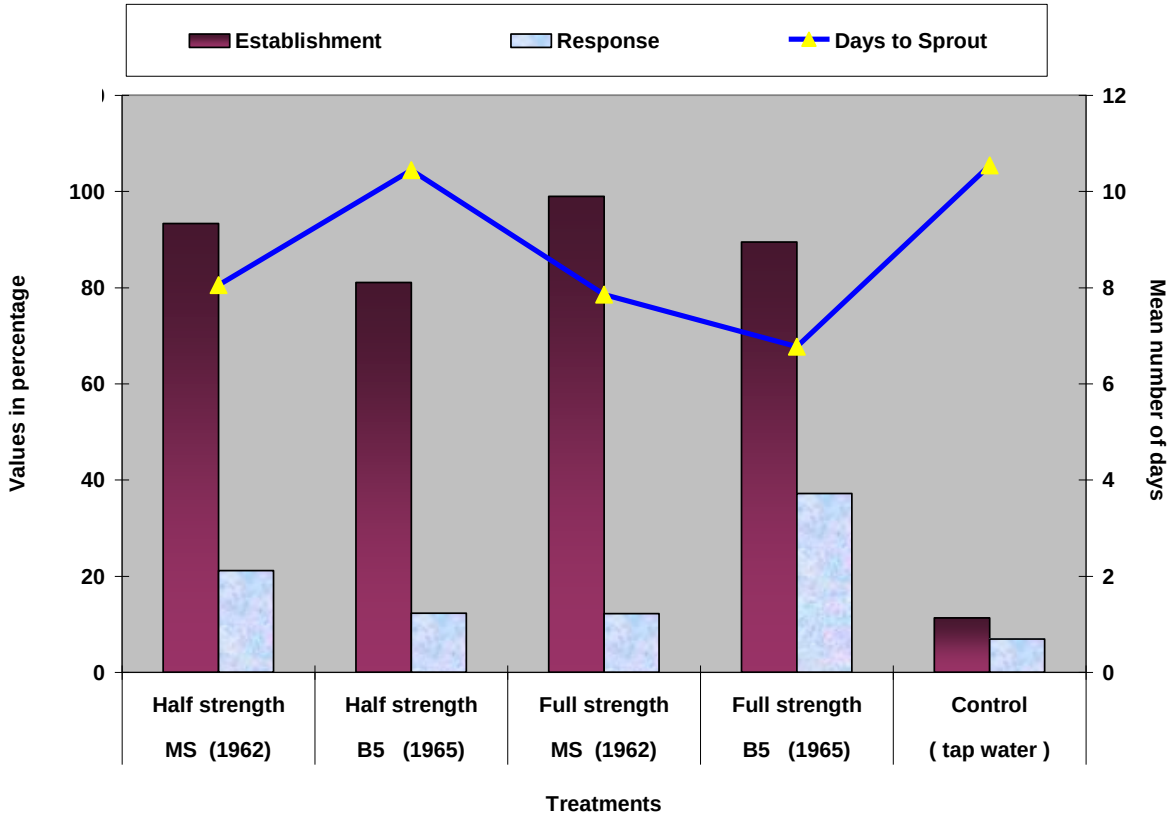


Fig 4: Effect of different basal media on growth and establishment

4.2.2. Effect of light and dark periods on establishment:

Various treatments of light and dark regimes for a period of 14 days after initial inoculation were tried to enhance the response and establishment percentage (Fig. 5).

Best establishment percentage (98.75 %) was recorded in treatment consisting of 4 days of complete dark followed by 3 days of continuous light regime followed by the same treatments repeatedly up to 14 days. Earliness for bud sprout (7.75 days) was observed in the same treatment with moderate percentage (94.14 %) as compared to other treatments. Two days of continuous light and 12 days of complete dark regime resulted in the second highest percentage (98.37 %) of establishment. The treatment consisting of 4 days of continuous light and 10 days of continuous dark regime resulted in lowest establishment percentage (90.94 %).

Maximum number of days (8.83) for bud sprouting was recorded in treatment No. 1 where the explants were grown under 2 days of dark condition followed by 12 days of light regime. However, the same treatment showed moderate response (95.41 %) and satisfactory establishment percentage (96.35 %).

These results are in accordance with reports by Parthasarthy *et al.* (2001), who described requirement of initial dark condition for only shoot emergence while further growth of Gerbera was observed under 12 hrs. light regime. Aswath and Biswas (2001) suggested that the illumination given at the end of the culture period may result in increased growth and development of explants as in the case of Anthurium as compared to the cultures kept in continuous dark or continuous illumination. Contrary to this, Ruffani and Massabo (2005) reported better shoot establishment in light than in dark.

Better establishment of explants under initial dark condition followed by light regime may be due to the higher sensitivity of juvenile tissues to light condition as suggested by Fotopoulos and Sotirophoulous (2007). Higher response under initial continuous light condition might be due to the result of mobilization of food material as a consequence of renewed enzymatic activity (Stiles and Cocking, 1969).

Results showed that the range of the percentage of establishment varied slightly (95 to 98) within various treatments. Same is the status with percentage response (90 to 98) and number of days to sprouting (7 to 9 i.e. only two days). This behavior indicates

more or less light independent nature of stevia shoots at establishment phase which could be related to its high regeneration capability and therefore may not require external stimuli such as light as suggested by Darkani *et al.* (2005).

However, more detailed experimentation on the effect of dark/light conditions on stevia establishment may be required to establish the reasons for this response.

Note: Treatments

T1) 2 days dark followed by 12 days light.

T2) 4 days complete dark followed by 10 days total light.

T3) 7 days complete dark followed by 7 days complete light.

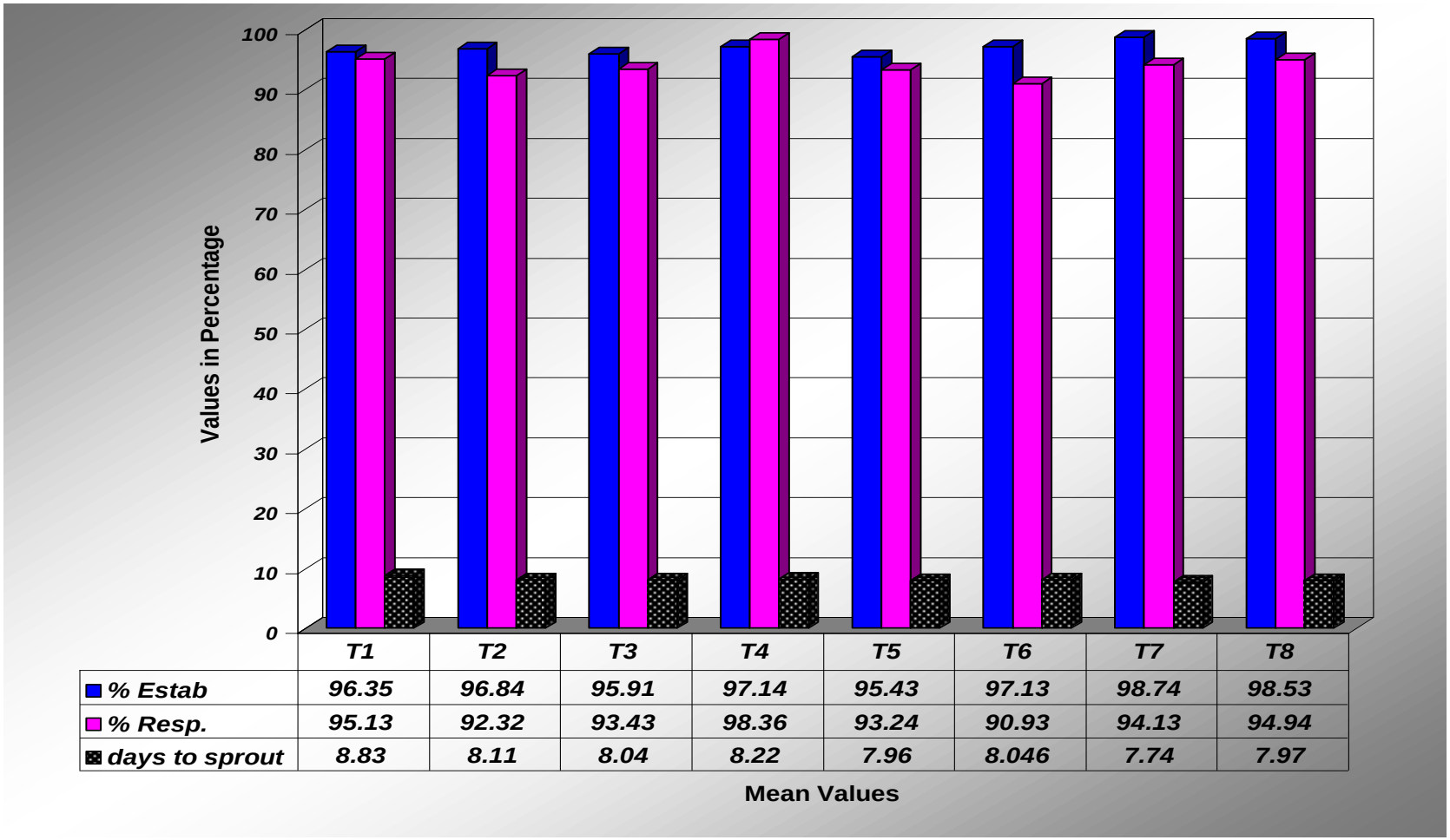
T4) 2 days complete light followed by 12 days complete dark.

T5) 4 days complete light followed by 10 days complete dark.

T6) 4 days complete light followed by 7 days complete dark followed by 3 days light

T7) 4 days complete dark regime followed by 3 days light regime again 4 days dark regime followed by 3 days dark regime.

T8) 4 days complete light regime followed by 3days dark regime again 4 days light regime followed by 3 days light regime.



	T1	T2	T3	T4	T5	T6	T7	T8
% Estab	96.35	96.84	95.91	97.14	95.43	97.13	98.74	98.53
% Resp.	95.13	92.32	93.43	98.36	93.24	90.93	94.13	94.94
days to sprout	8.83	8.11	8.04	8.22	7.96	8.046	7.74	7.97

Fig 5: Effect of light and dark periods on establishment of stevia explants

4.3. Multiplication Phase:

4.3.1. Effect of different cytokinins on formation and development of shoots:

To examine the effects of different cytokinins on multiple shoot formation, explants were cultured on MS medium supplemented with four cytokinins *viz.* BA, Kinetin, Thiadiazuron and Zeatin, at various levels (0.1 mgl^{-1} to 5.0 mgl^{-1}).

The results of the experiment designed to induce multiple shoots and subsequent growth are given in fig.6. It can be seen form the table that the nature of cytokinins affected not only the number of shoots but also the morphology of shoots. *In vitro* nodes produced multiple shoots in all the medium containing cytokinins. However, the number of shoots regenerated varied significantly at different levels of different cytokinins.

Among various treatments tried, highest degree of shoot proliferation was recorded on medium containing 2.0 mgl^{-1} BA with maximum number of shoots (35), nodes (122.1) and leaves (244.9). When the concentration of BA was increased from 2.0 $mgl^{-1}$to 5.0 mgl^{-1} , the number of explants forming shoots were decreased while, decrease in the concentration from 2.0 mgl^{-1} to 1.0 mgl^{-1}also showed the same trend (Plate 3).

Number of shoots, number of nodes and shoot length were found to decrease considerably in case of Kn containing media when concentration was increased from 2.0 mgl-^{1} to 5.0 mgl-^{1}. The cultured explants did not produce considerable number of shoots and development of shoots was not satisfactory on the Kn augmented medium as compared to BA or TDZ supplemented medium. The highest number of basal branching was obtained with 1.0 mgl^{-1}Kn or 2.0 mgl^{-1} of BA. Lowest number of branching at the base was found in control with only 2 shoots which was followed by 4 shoots in medium with 0.1 mgl^{-1}BA or 2.0 mgl^{-1} of Zn.

Eventhough, the least response was observed in control, the response of explants on Zn supplemented media at various concentrations (0.1 mgl^{-1} to 5.0 mgl^{-1}) was poor as compared to other cytokinins. The number of shoots were in the range of 16 to 22 while, the minimum number of nodes was 41.3 and 83 leaves whereas maximum number of nodes observed was 91 and 183 leaves on supplementation of Zn at the rate of 0.1 $mgl^{-1}$and 1.0 mgl^{-1}, respectively. On the other hand, the highest average shoot length (3.02 cm) was observed with 5.0 mgl^{-1} Zn which was found at par with the shoot length

observed on medium containing 0.1 mgl^{-1} BA. Moreover, BA and Zeatin also gave comparable results for average length. The shoot length ranged from 2.3 to 3.0 cm on Zeatin and 2.7 to 3 cm on BA of various levels.

In case of the number of nodes and leaves, the response of TDZ was comparable with BA. The highest number of nodes recorded was 114.1 with 229 leaves on TDZ (1.0 mgl^{-1}) supplemented medium as compared to 122 nodes and 245 leaves on 2.0 mgl^{-1} BA supplemented medium while, the basal shoots were lower (7) in TDZ than basal shoot branching on BA supplemented media. This shows the persistence of TDZ in media for higher time than BA.

Contrary to the present results, Tavazza *et al.* (2004) observed better shoot proliferation together with good quality of shoots of *Globe artichoke* (Asteraceae) using 2 mgl^{-1} Kinetin while, BA at 0.8 mgl^{-1} to 2.0 mgl^{-1} induced small shoots. Meancacimei *et al.* (1997) found zeatin more effective than BA or 2iP in terms of shoot proliferation in Olive micropropagation.

The present findings are contradictory to the reports of Morini *et al.* (2003) wherein, better performance of stevia shoots was observed by replacing BA with Kn in the medium. Further, the internodes were generally fairly pronounced on Kn than BA supplementation. In line with this, Mitra and Pal (2007) reported that Kn was more efficient in producing more number of healthy shoots than BA.

However, the present results are in agreement with findings of Mandal and Parthiraj (2006) and reported maximum response on medium supplemented with 2.0 mgl^{-1} BA alone while, increasing or decreasing the BA concentration resulted in decreased bud sprouting. Tadhani *et. al.* (2006) also reported better performance with BA than Kn at various concentrations for multiplication of stevia shoots.

The cytokinin like compound TDZ has also been used for micropropagation of many woody species, but has not been widely tested for members of Asteraceae. Erdag and Emek (2005) reported highest number of shoots per explant on medium supplemented with very low level (0.005 mgl^{-1}) of TDZ. The morphoregulatory effects of TDZ at low concentration may be related to its mode of action and attributed to its action in inducing cytokinin accumulation (Nhut *et al.,* 2007). In the present experiment

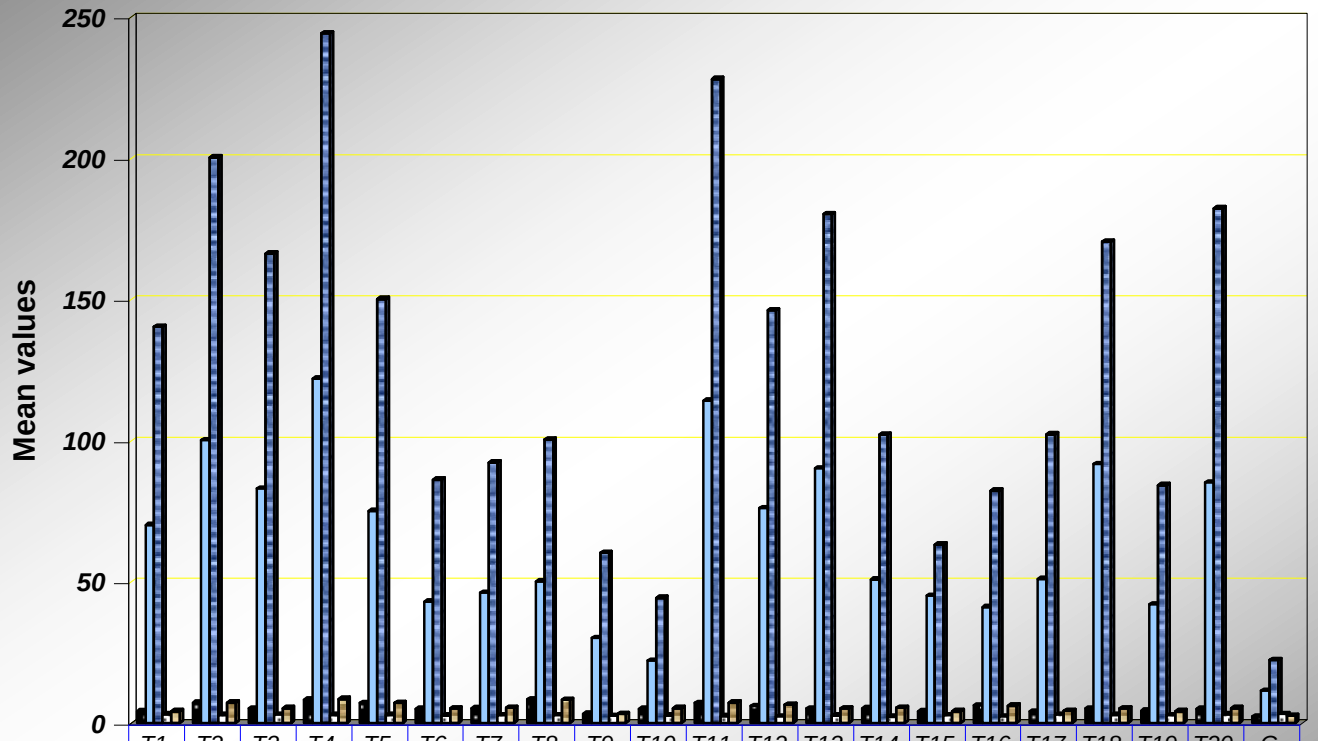
also it showed effectiveness at low concentration (0.1 mgl^{-1}). Visser *et al.* (2005) suggested TDZ as modulator of endogenous auxin which in synergism with cytokinins, may constitutes the inductive signal for different morphogenic expression. The same mechanism might be working in case of stevia multiplication and thus shoots are shorter and low in number but at the same time number of nodes and leaves were higher.

However, the best response observed with BA may relate to its selective stimulation of synthesis of polypeptide responsible for cell growth (Priyakumari and Sheela, 2005). The reduction in the frequency of regeneration at high doses of BA may be the result of the alteration in the physiological status of the explants or by way of interference of BA in other metabolic pathways at higher concentration as suggested by Srivastava *et al.* (1999).

The poor response of Kn than BA might be due to its control on certain enzymes in lesser extent. Sopory and Sharma (1990) reported the additive effects of BA on nitrate reductase while no effects of kinetin on the activity of nitrate reductase and Glutarate dehydrogenase.

The lesser effects of Zeatin may be related to its natural occurrence and therefore more prone to degradation by action of enzymes. It has been suggested that Zeatin might be converted into ribotide within 10 hrs. due to cleavage of the side chains and is affected differentially by cytokinin oxidase (Bhojwani, 1990).

Fig. No. : Effect of different cytokinins on multiple shoot formation and development



	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11	T12	T13	T14	T15	T16	T17	T18	T19	T20	C
No. of shoots	4.14	7.18	5.11	8.2	7.06	5.03	5.2	8.32	3.3	5.05	7.03	6.02	5.13	5.23	4.11	6.19	4.06	5.09	4.3	5.06	2.31
No. of nodes	70.1	100	83	122	75	43	46.1	50.1	30.1	22	114	76.1	90.2	50.8	45.1	41	51.1	91.7	42	85.2	11.4
No. of leaves	140	200	166	244	150	86.2	92.2	100	60.2	44.2	228	146	180	102	63.2	82.2	102	171	84.2	182	22.3
length of shoots	3.02	2.86	2.92	2.82	2.77	2.65	2.73	2.68	2.5	2.55	2.44	2.27	2.66	2.22	2.71	2.3	2.9	2.85	2.74	3.03	3.14
Branching at base	4.08	7.21	5.17	8.46	7.1	5.15	5.29	8.14	3.14	5.19	7.14	6.39	5.14	5.31	4.15	6.14	4.21	5.14	4.07	5.21	2.46

Treatments and corresponding values

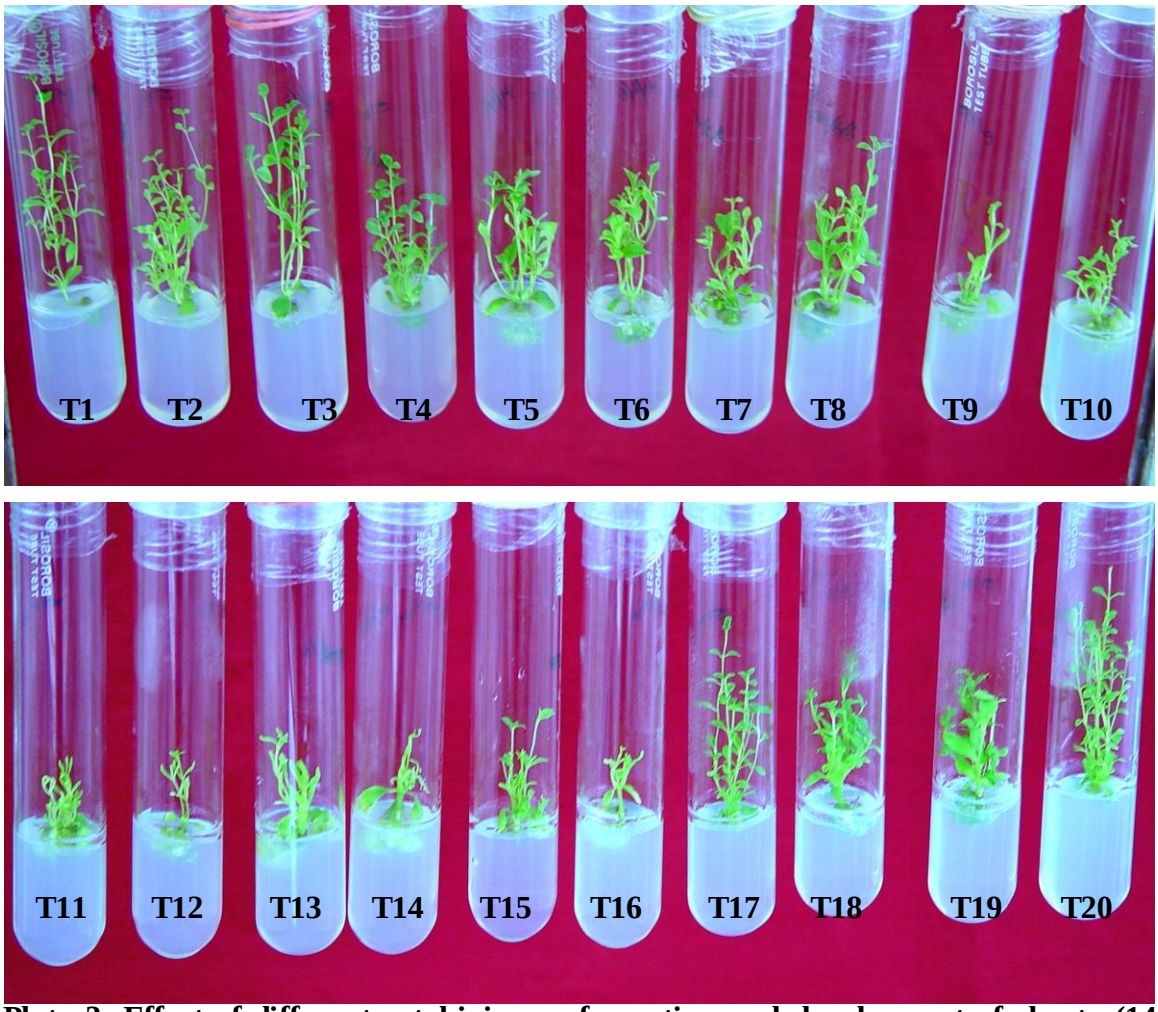


Plate 3: Effect of different cytokinins on formation and development of shoots (14 days after inoculation)

4.3.2. Effect of combination of BA and Kinetin:

After comparing various cytokinins at different concentrations, trials were conducted for further improvement in multiple shoot formation and development by using various combinations of two cytokinins viz., BA and Kn. These cytokinins were chosen on the basis of the previous experiments wherein BA was most responsive and kinetin the least responsive apart form Zn which was excluded due to its natural type. Significant differences were observed among different treatments tried. However, the maximum number of shoots (26) was observed with the lowest level of BA and Kn i.e. 0.1 mg^{-1} BA and 0.5 mgl^{-1} Kn. Highest numbers of nodes (14.0) and leaves (28) were also recorded in the same treatment. This was followed by medium containing 1.0 mgl^{-1} BA and 1.0 mgl^{-1} Kn, which showed the second highest number of shoots (25) with 13 nodes and 25 leaves (Table 1). The lowest number of shoots (12) and nodes (5.8) were observed on medium supplemented with 0.1 mgl^{-1} BA and 2.0 mgl^{-1} Kn. At par results were observed on treatment containing 1.0 mgl^{-1} BA and 0.5 mgl^{-1} Kn with 13 shoots, 6 and 12 number of nodes and leaves, respectively (Plate 4). On the other hand, medium supplemented with 1.0 mgl^{-1} BA and 1.0 mgl^{-1} Kn exhibited the highest shoot length (3.32 cm). However, same results were observed on media supplemented with 0.1 mgl^{-1} BA and 1.0 mgl^{-1} Kn, 1.0 mgl^{-1} BA and 2.0 mgl^{-1} Kn. Critical observations showed that at the medium concentration and same level of both cytokinins (0.1 mgl^{-1} BA and 1.0 mgl^{-1} Kn) resulted in higher number of nodes (13) and leaves (25) as compared to lower concentration and same level (0.1 mgl^{-1} BA and 0.1 mgl^{-1} Kn) or higher concentration and same level (2.0 mgl^{-1} BA and 2.0 mgl^{-1} Kn). However, combined effect of cytokinins at higher level (2.0 mgl^{-1}) resulted in lower shoot length as compared to other combinations containing the same level of BA and Kn. Over all, at lower concentration of BA (0.1 mgl^{-1}) and varied concentrations of Kn from lower to higher (0.5 to 2.0 mgl^{-1}) resulted in decreased number of shoots, nodes and leaves. At medium concentration of BA (1.0 mgl^{-1} of 0.2 mgl^{-1}) lowering or increasing the concentration of kinetin resulted in the decrease and increase in the number of shoots and nodes. However, gradual increase in kinetin concentration from 0.5 to 2.0 mgl^{-1} and of BA at higher level (5.0 mgl^{-1}) clearly

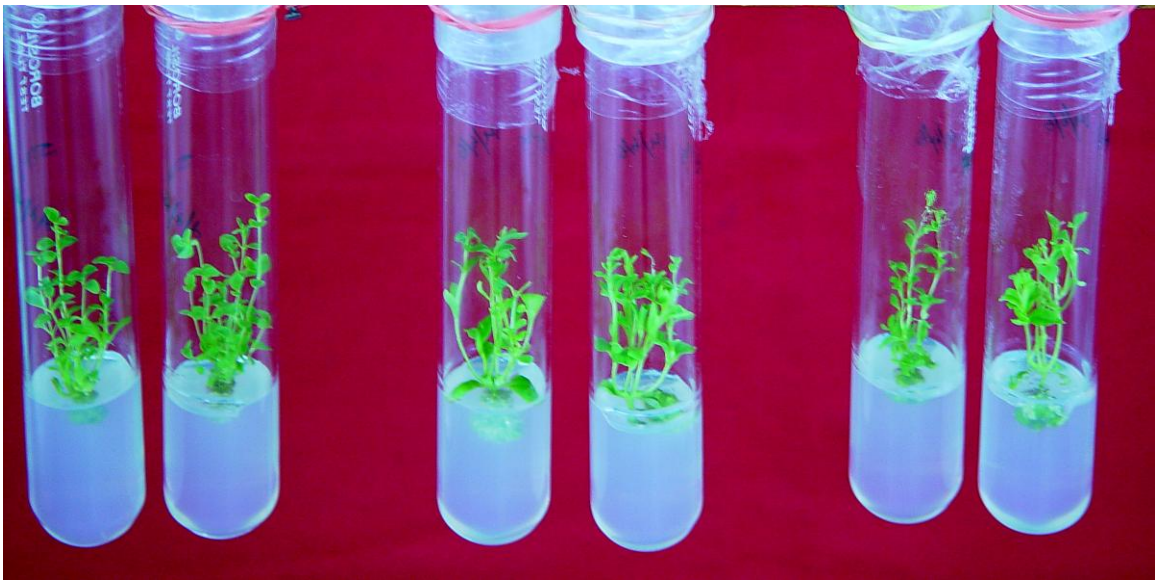
showed harmful effect on number of shoots, nodes and leaves. This indicates lower requirement of exogenous cytokinin.

Similar results were also reported by Baskaran and Jayabalan (2005), who showed that lower level of BA and higher level of Kn was beneficial in *Eclipta alba*. This might be due to the synergetic effect of cytokinins. BA and Kn were found to form stable complexes with enzymes and block the degradation of endogenous cytokinins. This reasoning can be applicable in the present response also indicating lower requirement of cytokinin. However, at higher concentration, over expression of the activity of enzymes might have been responsible for the decreased the bud growth (Kaminek *et al.*, 2005).

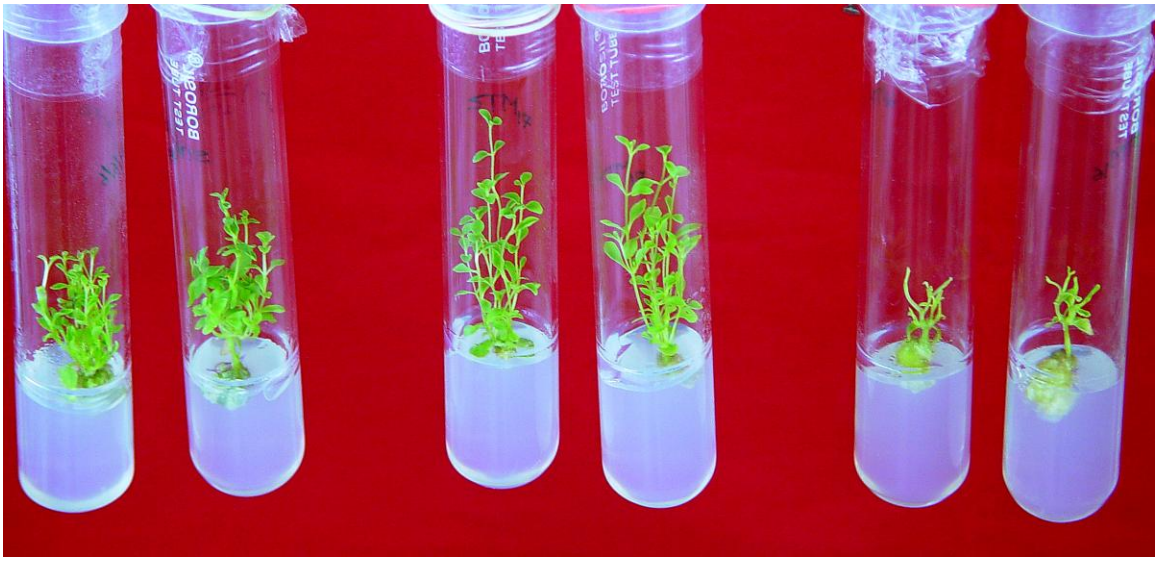
Outgrowth of axillary buds is well correlated with the cytokinins. It has been suggested that cytokinins independently regenerate the growth of axillary buds (Sato and Mori, 2001). This also found true for the present study. However, the combination of cytokinins failed to further improve multiple shoot formation and development as compared to the previous experiment.

Table 1: Effect of combination of BA and Kinetin on shoot multiplication development

Treatments	Cytokinin combination	No. of basal branches	No. of shoots	No. of nodes	No. of leaves	Length of shoots(cm)
T1	0.1 $mg1^{-1}$ BA + 0.5 $mg1^{-1}$ Kn	4.17	26.00	14.01	28.02	2.59
T2	1.0 $mg1^{-1}$ BA + 1.0$mg1^{-1}$ Kn	6.32	18.81	9.15	18.03	3.27
T3	2.0 $mg1^{-1}$ BA + 2.0$mg1^{-1}$ Kn	2.62	12.01	5.88	12.02	2.96
T4	4.0 $mg1^{-1}$ BA + 0.5 $mg1^{-1}$ Kn	3.11	13.01	6.00	12.08	3.09
T5	0.1 $mg1^{-1}$ BA + 1.0 $mg1^{-1}$ Kn	6.09	25.05	13.05	25.88	3.51
T6	1.0 $mg1^{-1}$ BA + 2.0 $mg1^{-1}$ Kn	5.04	17.03	8.01	15.84	3.24
T7	2.0 $mg1^{-1}$ BA + 0.5 $mg1^{-1}$ Kn	4.05	16.11	7.03	13.88	3.32
T8	4.0 $mg1^{-1}$ BA + 1.0 $mg1^{-1}$ Kn	4.02	14.01	6.03	11.99	2.58
T9	0.1 $mg1^{-1}$ BA + 2.0 $mg1^{-1}$ Kn	5.05	20.08	11.00	22.04	2.33
T10	1.0 $mg1^{-1}$ BA + 0.5 $mg1^{-1}$ Kn	7.01	22.01	12.18	24.03	2.14
T11	2.0 $mg1^{-1}$ BA + 1.0 $mg1^{-1}$ Kn	6.72	20.14	10.17	20.03	2.08
T12	4.0 $mg1^{-1}$ BA + 2.0 $mg1^{-1}$ Kn	4.17	17.15	8.88	18.03	1.69
T13	control	2.00	4.01	2.18	4.01	2.11
	SEm	0.18	0.88	0.24	0.31	0.12
	SEd	0.25	1.24	0.33	0.44	0.16
	CD	0.51	2.46	0.66	0.86	0.33
	CV%	9.51	12.44	6.63	4.35	10.61



T1 T2 T3 T4 T5 T6



T7 T8 T9 T10 T11 T12

Plate 4: Effect of combination of BA and Kinetin on multiple shoot formation

4.3.3. Effect of different media on multiplication:

The response of *in vitro* cultures to five different media (MS, B_5, LS, N & N and White's) was examined at full strength. The results showed that the degree of multiple shoot formation and development varied significantly with constitution of different media (Table. No. 2).

Among five media tested, B_5 medium was found as the best for number of shoots (6.7), number of nodes (3.24) and number of leaves (6.4) whereas, LS medium was proved superior for average length of shoots (2.3 cm). However, MS medium was found better in case of early bud sprouting (2.5 days) as compared to other media (Plate 5).

The least response of explants was observed on two media *viz.* White's as well as Nitsch and Nitsch medium where the number of shoots (2.5 and 3), number of nodes (1.08 and 1.22), number of leaves (5.3 and 5.8) and average length of shoots (1.14 and 1.07 cm) were respectively, obtained. Minimum number of days to bud sprouting was quite high on these two media (White's and N & N media) which ranged from 5 to 6 days as compared to other media tested where bud sprouting was observed within 2 to 3 days.

These results are contradictory to most of the reports on stevia where satisfactory response was obtained on MS medium (Toma *et al.*, 2000; Tadhani *et al.*, 2006 and Ibughin *et al.*, 2003). However, Miyagawa *et al.* (1986) preferred B5 medium for tissue culture of stevia, whereas LS medium was often used for organogenesis in stevia (Ferreira and Handro, 1987 and Tamura *et al.*, 1984). Use of Nitsch and Nitsch medium was reported previously by Patil *et al.* (1996); while White's medium was found to give comparable results in a study conducted on stevia micropropagation by Tamura (1984). The obvious reason for the least response of explants on White's medium could be its low number and concentration of salts (Razdan, 1990). While, poor performance of explants on Nitsch and Nitsch medium may be related to its higher Zn^{3+}, $H_3BO_3^-$ and lower Mg^+ content than other media tried (Hughes *et al.*, 1981). Ramkanthan and Jasrai (1997) observed mobilization and rearrangement of Zn^{+++} and Mg^{++} ions during sprouting of buds. Better results on B_5 medium might be because of higher nitrogen share

(12.50) of NO_3^-:NH_4^+ ions than on MS or LS medium (NO_3^-:NH_4^+ = 1.91) as suggested by Vidalee (1995). B_5 medium consists of moderate content of salts as compared to MS or LS medium which may be preferred for shoot proliferation (Bhojwani and Razdan, 1997).

Hughes (1981) suggested that the higher level of thiamine and exclusions of glycine and niacin could be the reason behind higher average length of shoots obtained on LS medium. The higher calcium level of MS and LS medium may have promoted the active growth of new forming shoots. George and Tripepi (2002) reported stunting of shoots and dieback of growing apices due to calcium deficiency in *Lewsia* cotyledons. However, MS or LS medium lacks or is lower in Na^+, Al^{3+} and Ni^{2+} (Amirouche *et al.*, 1985). Thus, the explants grown on MS medium showed fast bud sprouting but further growth was slower.

Table 2: Effect of different media on multiple shoot formation and development

Treatments	Media	Mean values				
		No. of shoots	No. of nodes	No. of leaves	Days to sprout	Length of shoots(cm)
T1	full strength B_5	6.36	10.47	22.11	2.43	2.07
T2	half strength B_5	6.90	13.54	27.11	2.63	3.50
T3	full strength LS	6.78	43.07	85.86	3.64	5.65
T4	half strength LS	5.77	20.42	39.87	4.53	3.18
T5	full strength MS	8.06	38.85	76.84	2.42	4.11
T6	half strength MS	7.10	22.13	41.69	3.05	3.06
	SEm	0.06	0.48	1.06	0.09	0.05
	SEd	0.08	0.67	1.50	0.13	0.07
	CD	0.17	1.37	3.07	0.27	0.15
	CV%	2.11	4.71	5.32	7.46	3.45

4.3.4. Selection of best medium for multiple shoot formation:

Total six treatments consisting of three types of media B_5, LS and MS media at full and half strength were evaluated for identifying the best medium among them to increase multiplication and development of stevia shoots.

Out of the six treatments tried, highest number of multiple shoots was recorded on full strength MS medium followed by half strength MS medium with an average of 8.0 and 7.1 shoots, respectively. Even though, the highest number of shoots were observed on MS medium the mean number of nodes and leaves were found to be significantly higher (43.0 and 85.8, respectively) on shoots regenerated on half strength LS medium. The highest average shoot length (5.6 cm) was recorded on full strength LS medium. The response of explants was early on full strength B_5 medium and required only two days to sprout (Plate 6). However, the same treatment was found least responsive in the case of mean number of nodes (10.4) and leaves (22.1). The lowest number of multiple shoots (5.7) was observed when full strength LS medium was used. Maximum number of days for shoot bud sprouting (4.5) was recorded in the case of half strength LS medium while, shortest shoot (2 cm) was found on full strength B_5 medium.

Overall, observations indicate that the formation of multiple shoots was significantly early and higher but with lower number of nodes, leaves and lower average length on MS medium than LS medium. Full strength B_5 media showed early bud sprouting but poor development of shoots as compared to MS or LS medium. However, at half strength, the multiple shoot formation took less number of days with higher average length on B_5 medium as compared to MS and LS medium. Half strength MS medium performed better than half strength LS or B_5 medium with comparatively higher number of shoots (7.1), nodes (22.1) and leaves (41.6).

These results are in agreement with the recent reports on stevia tissue culture (Mitra and Pal, 2007; Tadhani *et al.*, 2006; Mandal and Parthiraj, 2006) in which MS medium was preferred for multiple shoot formation. LS medium was used for organogenesis in stevia by Tamura and Handro (1988) whereas, lower salts containing medium *viz,* B_5 medium have been used for tissue culture of several crops

(Rout *et al.,* 2006) including stevia (Morini *et. al.,* 2003). However, detailed reports on comparative investigation of LS, MS and B_5 media is lacking in the case of stevia.

Nevertheless, the results are in accordance with the results obtained by Baskaran and Jayabalan (2005), who reported about the superiority of MS medium over B_5 medium for micropropagation of *Eclipta alba* (Asteraceae family member).

The probable reason for superiority of MS medium over B_5F medium might be related to its higher salt content. Ca^{2+}, NH^{4+} and Cl^- are present in considerably greater concentration in MS medium and LS medium than B_5 medium (Amirouche *et al.,* 1985). Moreover, B_5 medium contains nitrogen in the form of nitrate only, which needs more energy and carbon skeleton for its incorporation into cell as protein (Khetarpal *et al.,* 1999).

The higher elongation of shoots on full strength LS medium compared to MS medium seems to be related to its vitamin content. LS medium contains four times as much as thiamine than MS medium and omits pyridoxine, niacin and glycine (Hughes, 1981). Compared to this, the thiamine concentration is much higher (10 mg^{-1}) in B_5 medium (Mehta and Bhatt, 1990). The elongation of shoots was not better on full strength B_5 medium while, it was very good at half strength. This indicates that very high concentration of thiamine may be harmful for the multiplication and shoot development.

4.3.5. Optimization of medium concentration for multiplication:

Previous experiments showed that the MS medium performed better than other media and an experiment was carried out to optimize its concentration for further increasing multiplication potential of shoots. Half strength, 3/4 strength, 1/1 (full) strength and 1 ½ (1.5) strength of MS medium were examined to find out its optimum concentration for shoot multiplication and development.

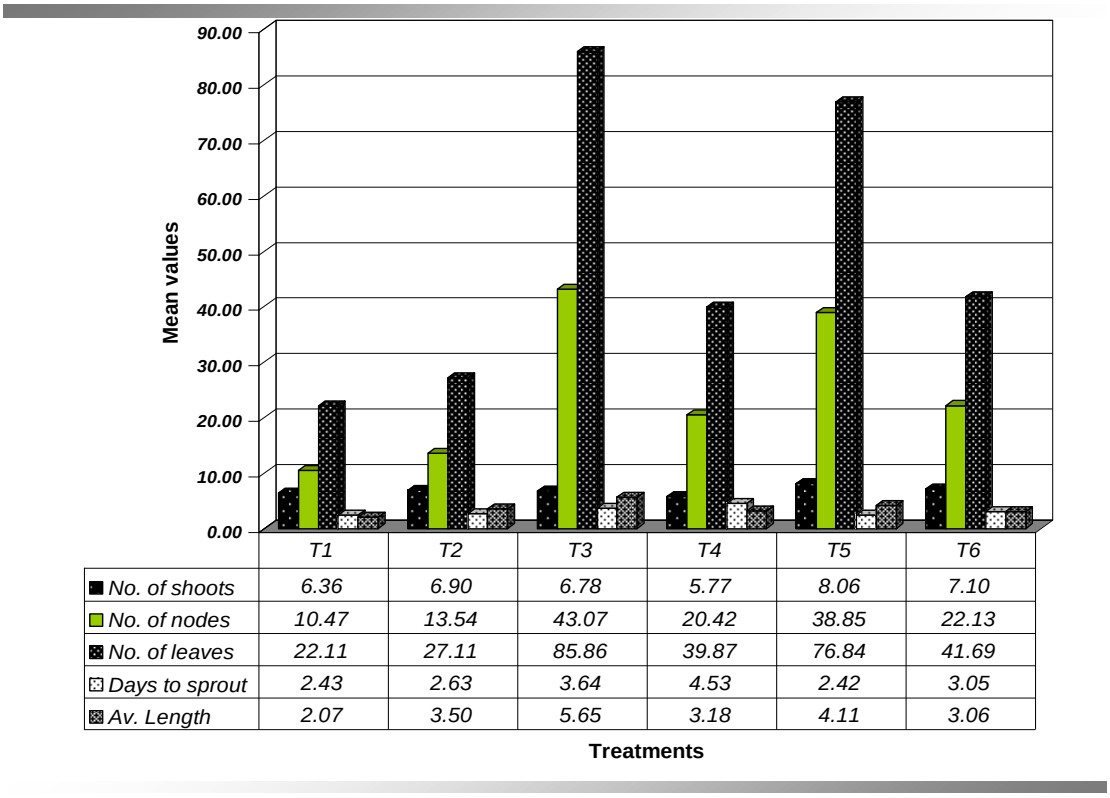
Comparison of shoot formation and morphological characters on various concentrations of MS medium showed expected results (Fig No. 7). The better results were obtained by increasing the salt strength upto certain limit (full strength) whereas,

very high concentration (1 ½ strength) brought about an adverse effects on growth parameters of cultivated shoots *viz.* length of shoots, number of shoots, number of nodes and number of leaves which were found decreasing (Plate No. 7).

Cultures grown on ¾ strength medium attained the highest average length of 4.7 cm. The same treatment also resulted in the highest number of shoots (8.1), maximum number of nodes (38.2) and leaves (80.5). Least response was observed on 1 ½ strength of MS medium, which resulted in lowest number of shoots (2.7), lowest number of nodes (2.0) and least number of leaves (4.2). Interestingly, the explants grown on 1 ½ strength MS medium showed stunted growth and necrosis. Overall, the cultures showed the following best trend for total number of shoots, number of nodes and number of leaves according for the concentration of MS medium from ¾ strength > 1/1 full strength > ½ strength > 1 ½ (1.5) strength. However, in case of average length, half strength MS medium was found superior to full strength of MS medium.

Though, the use of ¾ strength of MS medium was not a common concentration and therefore, the use of full strength MS medium for stevia tissue culture was reported by several authors (Mitra and Pal, 2007; Latha and Mukundan, 2003; Joi *et al*,2003). Present results are also in accordance with the findings of Osterc *et al.* (2005) who reported that ½ strength of MS medium resulted in better multiplication of chestnut nodal explants but increasing the $NH_4NO_3^-$ in the medium resulted in decreased multiplication after certain level and shoot elongation was better on half strength than full strength MS medium.

Higher concentration of MS salts were reported to be inhibitory for growth and multiplication as in the case of plant, *Laptinella nana* L. Compositeae family, (Carson and Leung, 1994). The adverse effects of 1 ½ strength of MS medium may be due to the increased salt concentration especially, Cl^- condition upto toxic level was supposed to inhibit NO_3^- uptake (Deane, 1990; Abrol, 1990) whereas, Yashita and Kohne (1990) suggested that at higher concentration, the molecules generally got accumulated in plant cells and might be injurious.



	T1	T2	T3	T4	T5	T6
No. of shoots	6.36	6.90	6.78	5.77	8.06	7.10
No. of nodes	10.47	13.54	43.07	20.42	38.85	22.13
No. of leaves	22.11	27.11	85.86	39.87	76.84	41.69
Days to sprout	2.43	2.63	3.64	4.53	2.42	3.05
Av. Length	2.07	3.50	5.65	3.18	4.11	3.06

Fig. 7: Selection of medium for shoot multiplication

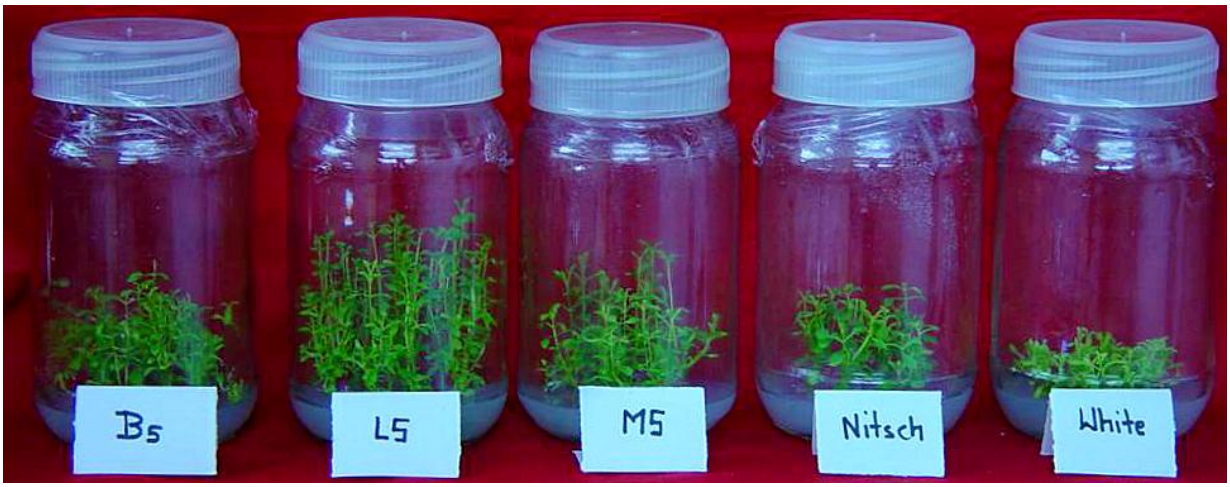


Plate No. 5 : Effect of different nutrient media on multiplication and development

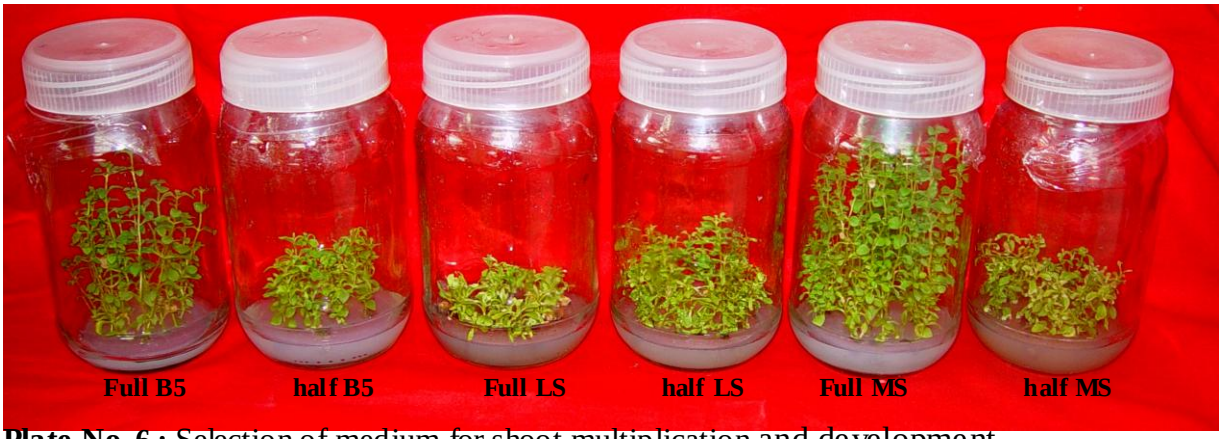


Plate No. 6 : Selection of medium for shoot multiplication and development

Plate No. 7: Optimization of medium concentration for multiplication

4.3.6. Positional effect of explants on mother plant:

In case of tree species, the initial position of isolated explants on its mother plant has been found to influence its morphogenic expression under *in vitro* conditions (Ramakanthan and Jasrai, 1997). Similar type of reports is scanty in case of herbaceous plants especially stevia. Hence, the present experiment was planned and the details of the experimental method are given in Table No. 3.8.7 . Among different explants tried, shoot initials isolated from the terminal/upper most position showed overall better multiplication and development with the highest number of shoots (49.3), more number of nodes (282.4) and higher number of leaves (562) along with higher length of shoots (4.68), as compared to other explants which could be due to its juvenile status. In *Gerbera jansanii*, also regeneration from juvenile plant parts was reported by Nhut *et al.*, 2007. Becerra *et al.* (2004) correlated the regeneration capacity to aging (as probably did the endogenous content of auxin) which could be associated with the loss of morphogenic capacity.

Contrary to the findings of Rao and Farrook (1997), who reported multiple shoot formation from only nodal cuttings taken from the middle of the young lateral branches, lowest number of nodes (138) and leaves (276) were recorded on explants excised from intermediary position on mother plant in the present study resulted in significantly higher number of nodes and leaves (254 and 506, respectively) but with lowest average length (4.15 cm) of shoots. However, the subapical portion of source plant resulted in the least number of (37.50) shoots (Fig. 8). These results support the hypothesis that the subapical and median buds, might be deficient in some specific enzymatic step but had capacity to store sufficient cytokinins. These events may be related to mobile hormones which are presumably synthesized in shoot tip and its young leaves or in the roots and transported downwards (Auxins) or upwards (Cks) in the stem (Pilate *et al.*, 1989) which may lead to differential cell division and development response in differentially aged explants. Difference in cell division and bud regeneration ability between juvenile and adult plant in *in vitro* has been reported by Stoutemyer and Britt (1965).

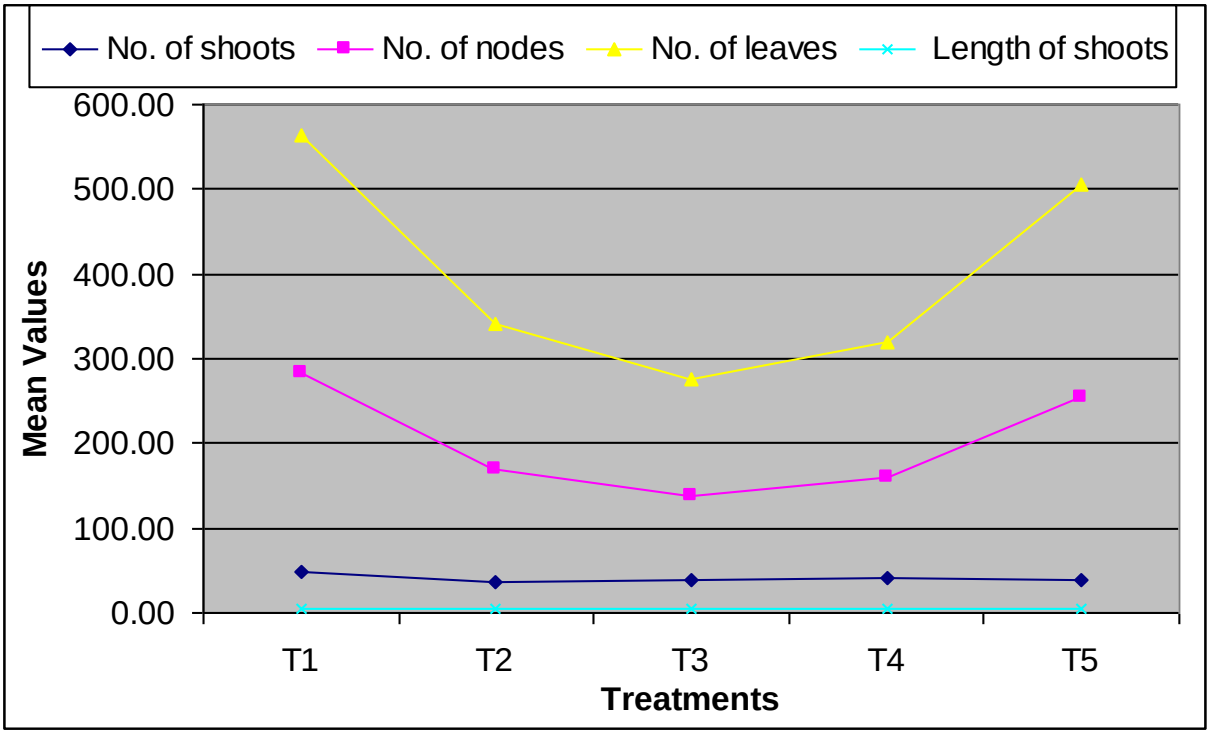
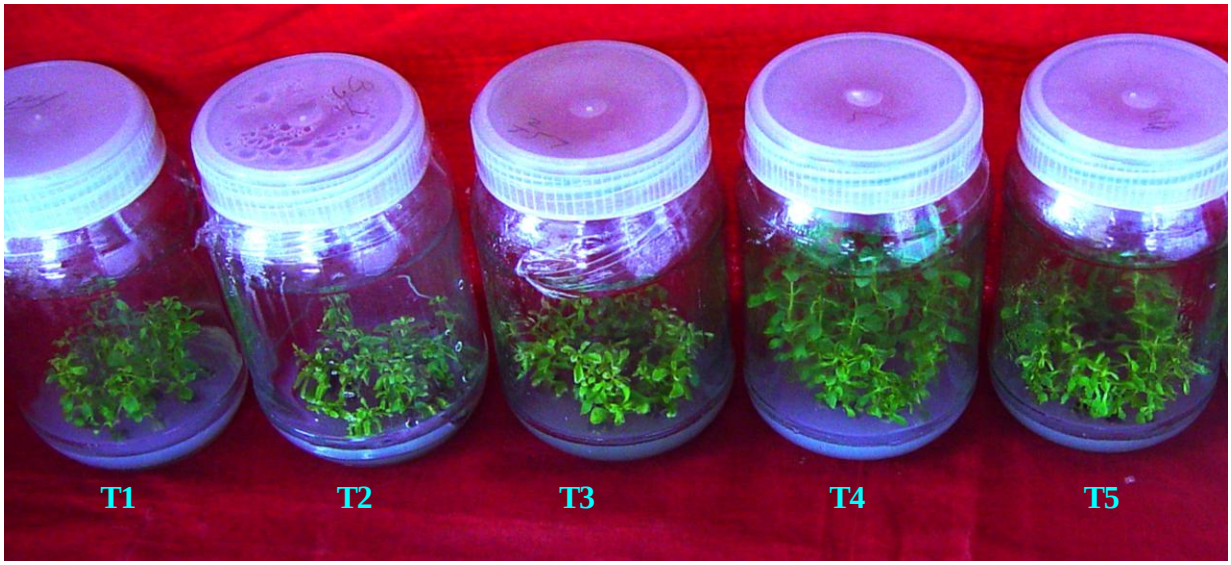


Fig. 8: Positional effect of explants on mother plant (Values represents number of various characters observed in all shoots in single bottle)



T1 = Uppermost node **T2 = Subapical node** **T3 = Mediary node**

T4 = second lower node **T5 = Lowermost or Basal node**

Plate 8: Positional effect of explants on mother plant

4.3.7. Effect of number of nodes on multiplication:

To find out the effect of retention of nodes on shoot formation and multiplication, a comparison was made in five types, explants differing in number of nodes viz. single node, shoot with 2 nodes shoot with 3 nodes shoot with 4 nodes and shoot with 5 nodes. The results are presented in Plate 9. The total number of shoots, nodes and leaves were increased as the number of nodes was increased in explant. The explant consisted of single node gave rise to lowest number of shoots (4.5), nodes (2.3) and leaves (4.8) while, the explant having two nodes resulted in slightly higher number of shoots (6.8) with 2.8 node and 5.5 leaves. Shoots having 3 nodes showed the highest number of shoots (7.8) as compared to other explants with second highest number of leaves (11). The shoot consisted of four nodes resulted in lower number of shoots (6.5) but higher number of nodes (5.5) and leaves (10.6) than explants with 2 nodes whereas, highest number of nodes (6.3) and leaves (13) were observed with shoots having 5 nodes. The same explant, showed total 7 shoots which was lower only to shoots produced by explants with 3 nodes. However, if ratio of the number of shoots per node is considered, the single nodal explant performed better than other explants with 4.5 shoots produced per node while, the lowest number of shoots per node (13) was recorded in shoots with 5 nodes.

It was interesting to note that in all cases the formation of shoot was started first at basal node followed by upper most node (Plate No. 9). This indicates that locally synthesized cytokinins might have involved in the outgrowth of the axillary bud as also observed by Kaminek *et al.* (2005). However, the present data showed contradictory trend to the findings of Ramakanthan and Jasrai (1997) who reported simulation of growth in buds of upper and lower portions of *Emblica officinalis* shoots.

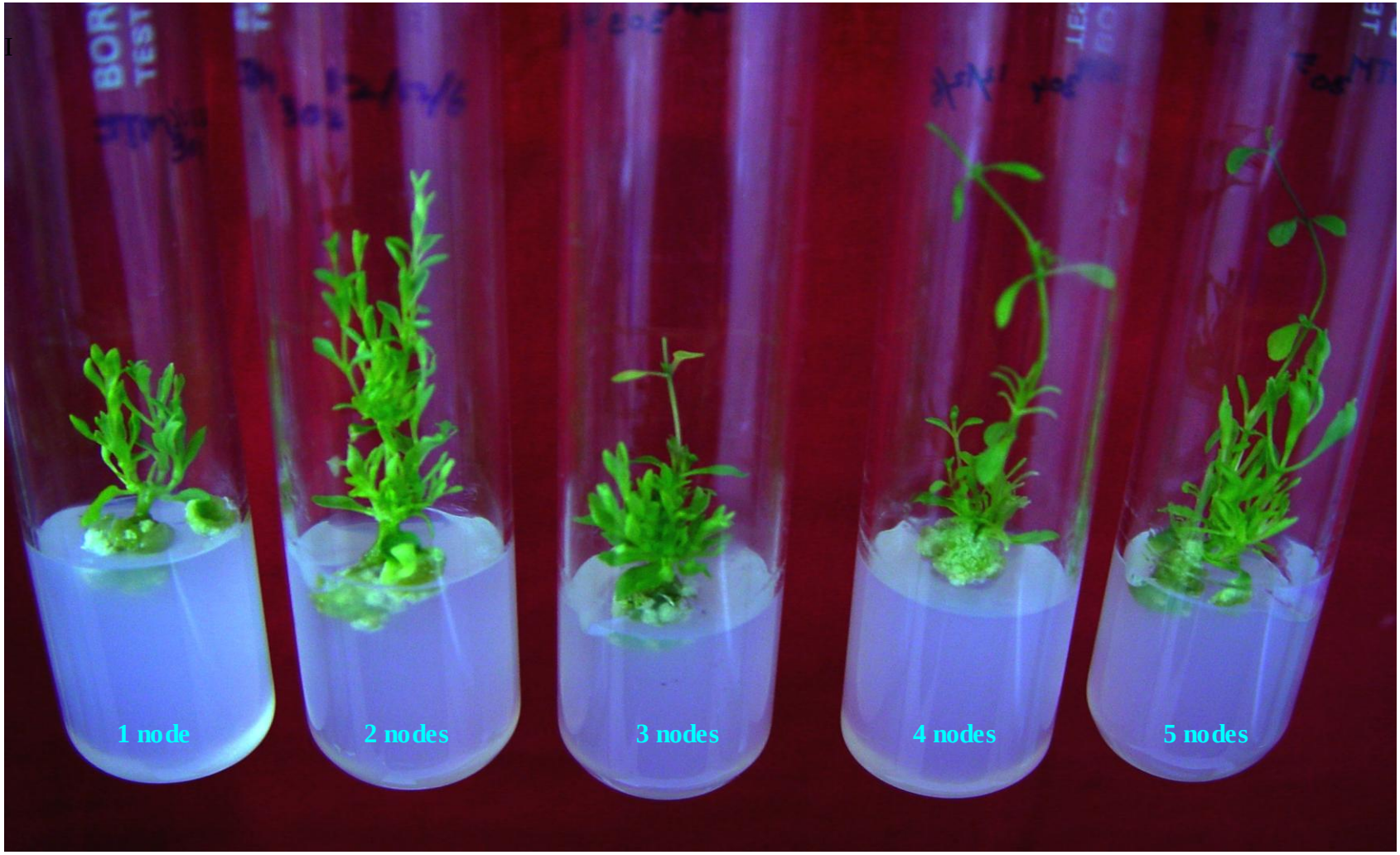


Plate 9: Effect of number of nodes on multiplication

4.3.8. Effects of different intensities of light on multiplication:

Fwrnkrage *et al.* (1990) reported that light intensity has a significant effect on shoot multiplication and *in vitro* growth of plants. However, very scanty reports are available related to the effects of light intensity on stevia micropropagation. Therefore, trials were conducted to evaluate the performance of *in vitro* grown shoots under three different light intensity regimes including low light intensity (20 µmol m^{-2} s^{-1}), medium light intensity (40 µmol m^{-2} s^{-1}) and high light intensity (60 µmol m^{-2} s^{-1}).

Table No. 3. shows that the explants grown under lowest light intensity (20 µmol m^{-2} s^{-1}) resulted in lower overall multiplication (2.6 shoots) and development of shoots and highest intensity (60 µmol m^{-2} s^{-1}) resulted in higher multiplication of shoots (4.68) while, medium light intensity (40 µmol m^{-2} s^{-1}) resulted in significantly higher multiplication (6.4) of shoots and highest number of nodes (16.5) and leaves (32). Similar trend was observed for the days to bud sprouting in this study wherein minimum period of two days was required for sprouting as observed under medium light intensity. Sprouting took maximum number of days (4.3) under lower light intensity. However, visual observation (data not shown) suggested that the lowest light intensity was beneficial for increased elongation of shoots (Plate No. 10) followed by medium light intensity and higher light intensity. These observations are contradictory to the findings of Karhu and Hokala (1990) wherein, medium level of light intensity was found to have no significant effect on shoot development whereas, Alphonse *et al.* (2002) observed that exposure of *Arthichoke* (Asteracae member) cultures to lower light intensity (20 µmol m^{-2} s^{-1}) was more suitable for shoot elongation and multiplication along with higher number of leaves as compared to those produced under high light intensity (60 µmol m^{-2} s^{-1}). This may be due to varying genus effect. However, the results are in complete agreement with the reports of Sevenson (2002) on *Aristolachia mansheriensis* wherein, lowest light intensity resulted in lowest multiplication rate and medium light intensity resulted in higher multiplication. The inhibition of growth by high light intensity might have been due to low IAA oxidase activity (Seibart *et al.,* 1975). According to Puargpaka *et al.* (2001), plant growth is related to the function of growth hormone like

auxin, which is sensitive to high light intensity and cytokinins act in concert with auxin in plant tissue culture. However, the higher growth and multiple shoot formation under medium light intensity might not be directly to the hormonal balance but may be the result of evolution of CO_2 in optimum concentration above the agar (Pierik, 1989). Increased elongation of shoots under low light intensity may be related to increased synthesis of endogenous gibberellins because gene expression for gibberellin biosynthesis is found to be regulated by light intensity (Sunduwhar, 2006).

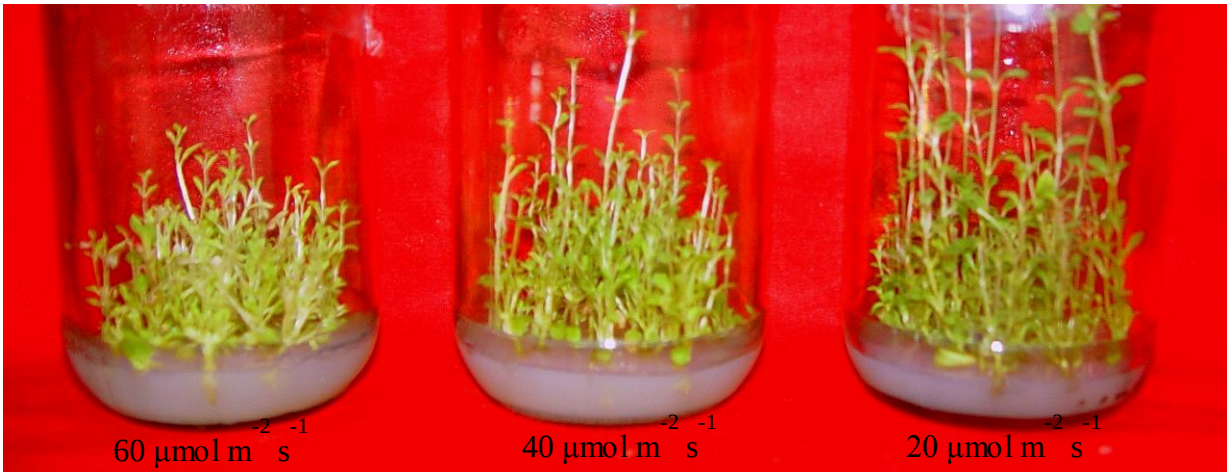


Plate 10: Effects of different light intensities on multiplication and growth

Table 3: Effects of different light intensities on multiplication and growth

Treatments	Days to Sprouting	No. of Shoots Per Explant	No. of Nodes Per Explant	No. of Leaves Per Explant
60 µmol m^{-2} s^{-1}	4.28	12.02	59.95	120.48
40 µmol m^{-2} s^{-1}	2.36	19.23	68.63	136.09
20 µmol m^{-2} s^{-1}	3.51	16.60	46.22	92.66
SEm	0.12	0.26	0.28	0.18
SEd	0.17	0.36	0.39	0.25
CD	0.36	0.78	0.84	0.53
CV%	8.68	3.95	1.17	0.37

4.3.9. Effect of different photoperiod on multiplication and development of stevia shoots:

The duration of light/dark periods are known to exert marked effects on differentiation, growth, development, and reproduction by profoundly influencing the general metabolic activities in plants. However, little is known about the effect of photoperiod on *in vitro* culture of stevia. Therefore, present experiment was carried out to find out photomorphogenic effects of various light/dark regimes. For this purpose, different photoperiods including 0 hrs. light /24hrs. dark, 24 hrs. light / 0 hrs. dark ,16 hrs. light / 8 hrs. dark, 8 hrs. light / 16 hrs. dark and 12 hrs. light /12hrs. dark periods were tried. The results are presented in fig. 9.

Maximum number of multiple shoots (56) was observed when the explants were grown under 8 hrs. light/ 16 hrs. dark period. Comparable results were obtained when explants were exposed to 12 hrs. light /12 hrs. dark regime (54) whereas the complete dark regime resulted in the lowest number of multiple shoots (38). The highest number of countable leaves (404) was obtained in 8 hrs. light/ 16 hrs. dark period followed by 12 hrs. light /12 hrs. dark regime, while lowest number of leaves (218) were observed under complete dark period. However, the highest shoot length (longest internodal length) was recorded under 12 hrs. light period followed by 8 hrs light/ 16 hrs. dark period (Plate No.11) The duration of light dark period also affected the time taken for bud sprouting. Minimum number of days to bud sprouting was noted (1 day) under 8 hrs. light/ 16 hrs. dark period. The increase in the duration of light period (8 hrs. ,12 hrs. and 16 hrs.) showed an increasing trend (1, 2 and 4 days) for days to sprouting whereas complete dark or complete light dark regime gave medium type of response (2 days) and showed inhibitory effect of long light hours. This could be explained by the photoinduction control due to interaction of light and auxin /cytokinin.

As expected, the shoot grown under complete dark exhibited etiolation. These findings are in accordance with the reports made by Bhatia and Ashwath (2005). They reasoned this response by stating that the light acts as main regulator of the ultra structural organization of plant photosynthetic apparatus. Thus, in the absence of light, proper chlorophyll development was hampered and therefore shootlets were etiolated

whereas, reduced multiplication and development might be related to the release of methane and ethylene due to dark condition (Sudarson *et al.*,2003) .

Xu *et . al.*(1997) stated that the stem elongation under short day conditions in rosette plants as a mediation by GA_3. The same could be the reason for higher elongation or internodal length of shoots in 12 hrs. light or 8 hrs. light periods indicating clear photoperiodic control and relation with gibberellins. But the overall development was not vigorous due to similar length of light and dark periods where the probability of light stimulated synthesis of enzymes and increased metabolic activities were compensated by reverse processes during dark periods (Sawhnay and Naik, 1990). The highest multiplication and development under 8 hrs. light / 16 hrs. dark period might be the result of better nitrate assimilation. Several reports established the increased availability of NO_3^- under light (Sopory *et. al.* 1994). Moreover, increased synthesis and accumulation of different proteins on exposure to short light period after a long dark period or vise-versa is reported by Mullet (1998).

However, similar results were not obtained in the present study with increased light durations (12 hrs.,16 hrs. and 24 hrs.) which may be due to the interference of endogenous hormonal balance and light for longer periods than the critical limit (Stiles and Cocking,1969)

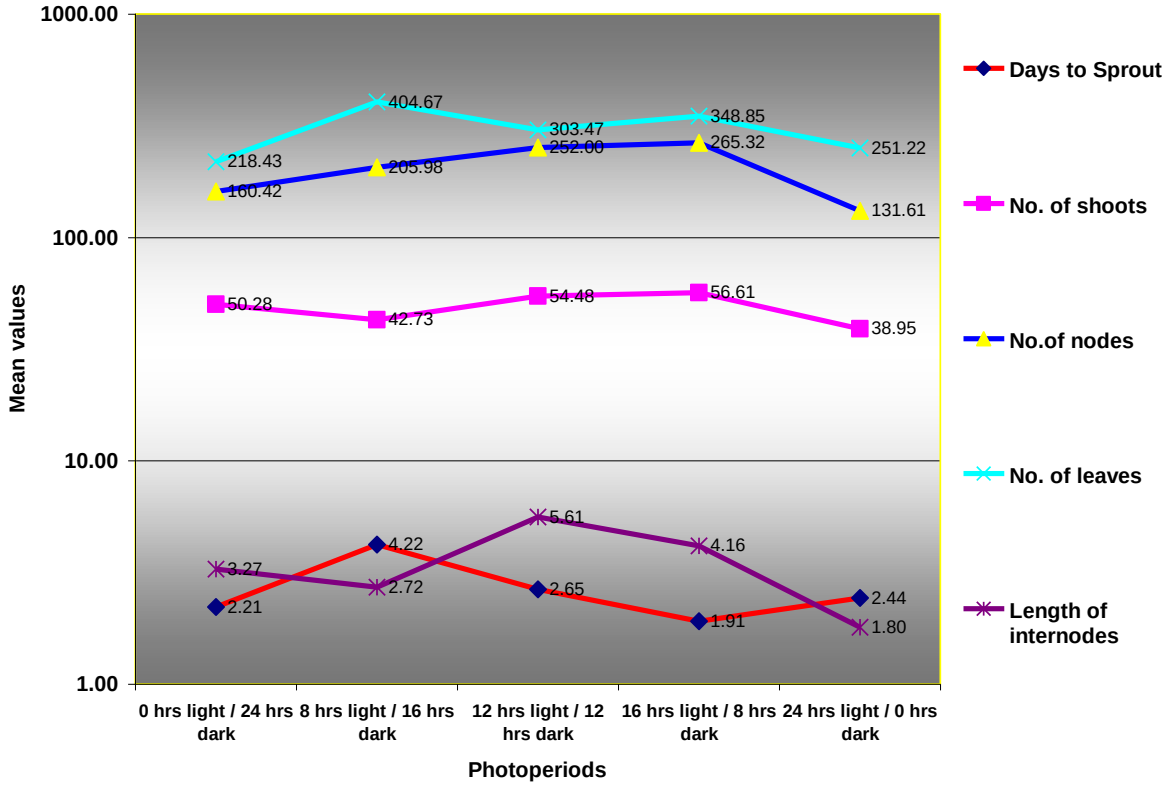


Fig 9: Effect of different photoperiod treatments on growth and multiplication

Plate 11: Effect of different photoperiod treatments on growth and multiplication (14 days after of inoculation)

4.3.10. Effect of number of subculture/multiplication cycles on multiplication:

To find out relationship between subculture passage and the morphogenic potential of plantlets, an experiment was carried out to assess the effect of number of subculture on multiplication using higher level of BA i.e. 2.0 mgl^{-1} as well as lower level of BA i.e. 0.5 mgl^{-1}. Results showed the superiority of lower level of BA (0.5 mgl^{-1}) over higher concentration (2.0 mgl^{-1}) during various subculture passages (Table No. 4).

Higher number of shoots (79714) was obtained on medium supplemented with 0.5 mgl^{-1}BA after sixth subculture as compared to 5479 shoots obtained on medium supplemented with 2.0 mgl^{-1} BA. However, multiplication rate (3 to 6) was found higher on 2.0 mgl^{-1} than 0.5 mgl^{-1} BA (2.5 to 4) upto second multiplication cycle. On the other hand, multiplication rate was higher (6.03) on 0.5 mgl^{-1} BA than 2.0 mgl^{-1} BA (4.03) after sixth subculture. The cultures of both level of BA showed increase in multiplication rate i. e. 2.5 to 6 upto fourth passage. During fourth passage cultures showed highest multiplication rate (6 to 6.5). The cultures grown on medium fortified with 2.0 mgl^{-1} BA resulted in gradual decrease in multiplication rate (6 to 4) after 4^{th} passage whereas, shoots cultured on 0.5 mgl^{-1} BA showed no such phenomenon and multiplication rate remained stable even after sixth subculture. None of the cultures showed any sign of variability during the course of study. This indicated that repeated subculturing could produce a large number of *in vitro* stevia plants.

Similar results were reported by How and Key (2000) who obtained satisfactory number of shoots on medium supplemented with 0.5 mgl^{-1} BA after first subculture. Tiwari *et al.* (2001) also reported the increase in multiplication rate with the number of subculture passage. Samanthi *et al.* (2004) reported that single exposure to low concentration of BA reprogrammed the development throughout the shoot apex. The decrease in multiplication rate after the fourth subculture on 2 mgl^{-1} BA might be related to gradual decrease of ribotide during subculturing passage (Sreedavy *et al.*, 2002).

Table 4: Effect of number of subculture cycles on multiplication

Media	Subculture passage	Duration in each passage	No. of shoots inoculated	No. of shoots produced	Multiplication rate
MS + 2 mgl^{-1}BA	I	21 days	6	18	3
	II	21 days	18	63	6
	III	21 days	63	378	6
	IV	21 days	378	2268	6.5
	V	21 days	2268	13698	6
	VI	21 days	13698	54794	4
MS + 0.5 mgl^{-1} BA	I	21 days	6	15	2.5
	II	21 days	15	60	4.08
	III	21 days	60	366	6.1
	IV	21 days	366	2212	6.03
	V	21 days	2210	13219	5.98
	VI	21 days	13219	79446	6.01

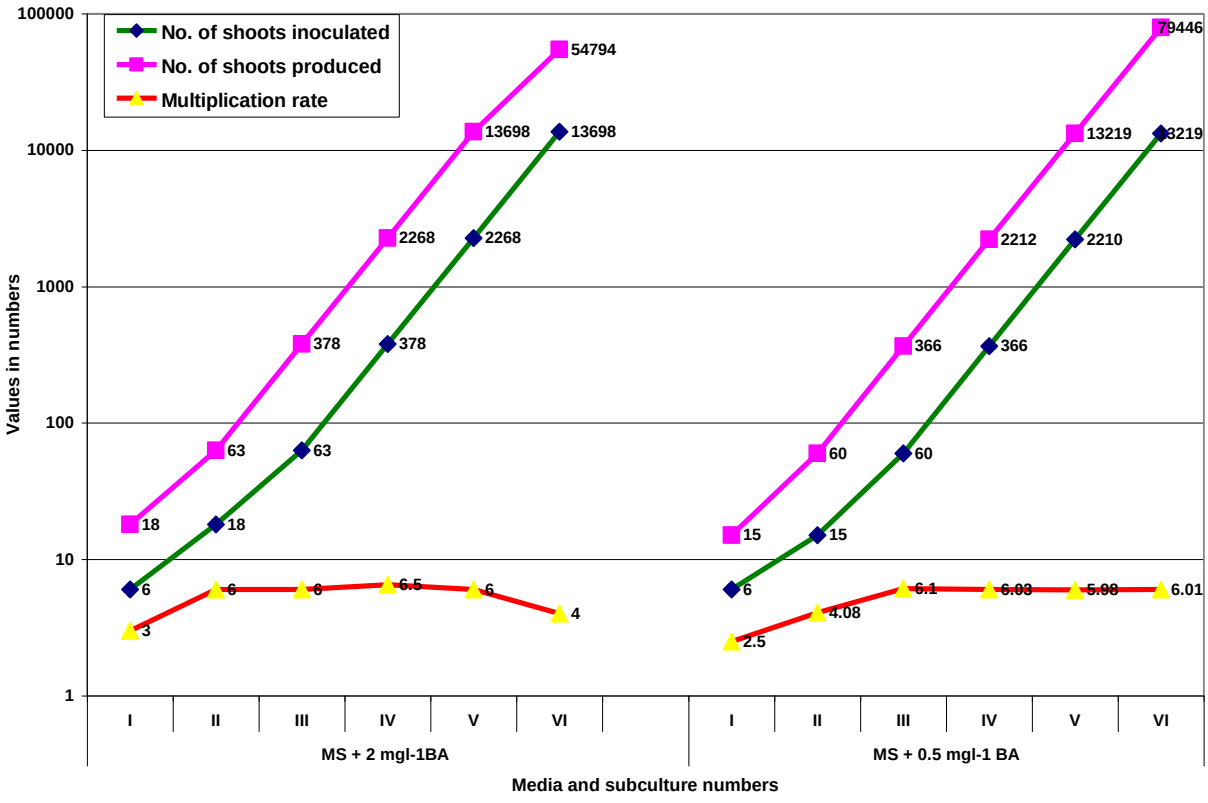


Fig 10: Effect of number of subculture cycles on multiplication

4.3.11. Influence of different sugars on multiplication of shoots:

Five types of sugars i.e. Fructose, Glucose, Lactose, Maltose and Sucrose at various concentrations (1 % to 5 %) were tried to evaluate their influence on shoot multiplication and the results are presented in Table No.5.

Among different treatments tried, 3% sucrose resulted in significantly higher number of shoots (54.3) while the lowest number of shoots was observed on media supplemented with 5 % lactose. Highest average shoot length (5.65 cm) was also observed with 3 % sucrose which was followed by 1 % fructose, whereas 5 % lactose gave an average shoot length of 1.57 cm only which was the least and less than the control (treatment without sugar).The treatment consisted of maltose and glucose was found less beneficial than sucrose and fructose (Fig. 11) at various concentrations. The results were contradictory to the reports of Cuenoa and Vietez (2000). However, it is in total agreement with the findings of Baskaran and Jayabalan (2005). Sucrose has been preferred carbon source in tissue culture of stevia (Bondarev *et. al.* 2003) while fructose was used occasionally for plant tissue culture (Silva.*et al.*, 2005). Better response on sucrose supplemented medium might be due to the efficient uptake of sucrose across plasma membrane of the plant cells while slow growth of the cultures on fructose containing medium might be the result of the inhibition of glycolysis by fructase enzyme or its degraded products (Oka and Ohyama,1982).

The shoots cultured on sucrose fortified media also seemed to be healthier than cultures grown on fructose supplemented media which showed the highest number of nodes (261) and leaves (522) (Fig. 11).The difference in response could not be directly linked to the carbohydrate nutritional aspects, but with its osmotic contribution which was clearly observed at higher levels (4 % to 6 %) of carbon sources that resulted in decreased shoot formation and multiplication. This may be explained as the capacity of the *in vitro* plants to absorb, metabolize, assimilate and utilize the carbon source at a low concentration due to change in osmotic potential of the media (Pritchard *et al.*, 1991).

Less influence of glucose on multiplication of shoots may be directly related to its monosaccharide nature due to which it might be more easily degraded. However, effects of maltose for green plant production has been explained as an effect of medium

osmolarity (Zhou *et al.*, 1991) whereas the poor response on lactose supplemented media may be related to lower activity of the enzyme *β*- galactosidase (Fowler *et. al.*, 1982)

Results also showed that the presence of sugar in the nutrient medium is an essential factor for multiplication and development of stevia shoots *in vitro* (Fig. No. .) . The growth was markedly influenced by the presence of sugar even at concentration as low as 1 %. Interestingly, shoot formation was also seen on the nutrient media without any type of sugars. Amtrosio and Demelo (2004) explained that the occurrence of such phenomenon because of the fact that carbohydrates supplemented to the plant tissue during the previous culture phases might have got accumulated in sufficient amounts to start the growth.

Overall frequency of multiple shoot formation and development showed variation on the different sources of carbon. This shows that the formation of multiple shoots was highly influenced by type and concentration of sugars.

Table 5: Influence of different sugars on multiplication:

Treatments code	Sugars	No. of shoots	No. of nodes	No. of leaves	length of shoots(cm)
T1	1% Fructose	44.22	190.51	380.79	5.33
T2	1% Glucose	43.07	160.58	321.79	3.63
T3	1% Lactose	41.05	151.38	301.92	4.57
T4	1% Maltose	25.78	94.14	186.70	4.37
T5	1% Sucrose	45.55	222.93	441.17	4.23
T6	2% Fructose	49.53	188.26	376.26	3.91
T7	2% Glucose	50.64	184.55	370.61	4.83
T8	2% Lactose	42.42	86.06	173.28	3.14
T9	2% Maltose	32.99	200.02	401.75	4.91
T10	2% Sucrose	50.35	163.02	324.71	4.16
T11	3% Fructose	37.31	81.19	161.97	2.84
T12	3% Glucose	37.69	108.55	216.30	3.55
T13	3% Lactose	32.71	88.28	176.05	2.68
T14	3% Maltose	38.17	80.62	160.23	3.42
T15	3% Sucrose	54.30	261.09	522.10	5.65
T16	4% Fructose	33.43	196.46	397.92	4.86
T17	4% Glucose	23.39	150.13	300.11	3.21
T18	4% Lactose	31.37	74.28	148.47	1.92
T19	4% Maltose	40.61	158.30	319.21	1.59
T20	4% Sucrose	45.20	251.01	501.18	2.62
T21	5% Fructose	22.94	110.54	225.27	3.53
T22	5% Glucose	22.74	73.08	204.56	2.14
T23	5% Lactose	22.60	101.81	208.37	1.57
T24	5% Maltose	49.14	98.03	193.36	2.45
T25	5% Sucrose	45.11	241.38	482.68	3.83
T26	Control (no sugar)	13.27	24.99	43.84	1.86
	SEm	2.26	0.96	6.36	0.11
	SEd	3.20	1.36	9.00	0.15
	CD	6.33	2.69	17.81	0.30
	CV%	14.76	1.64	5.37	7.57

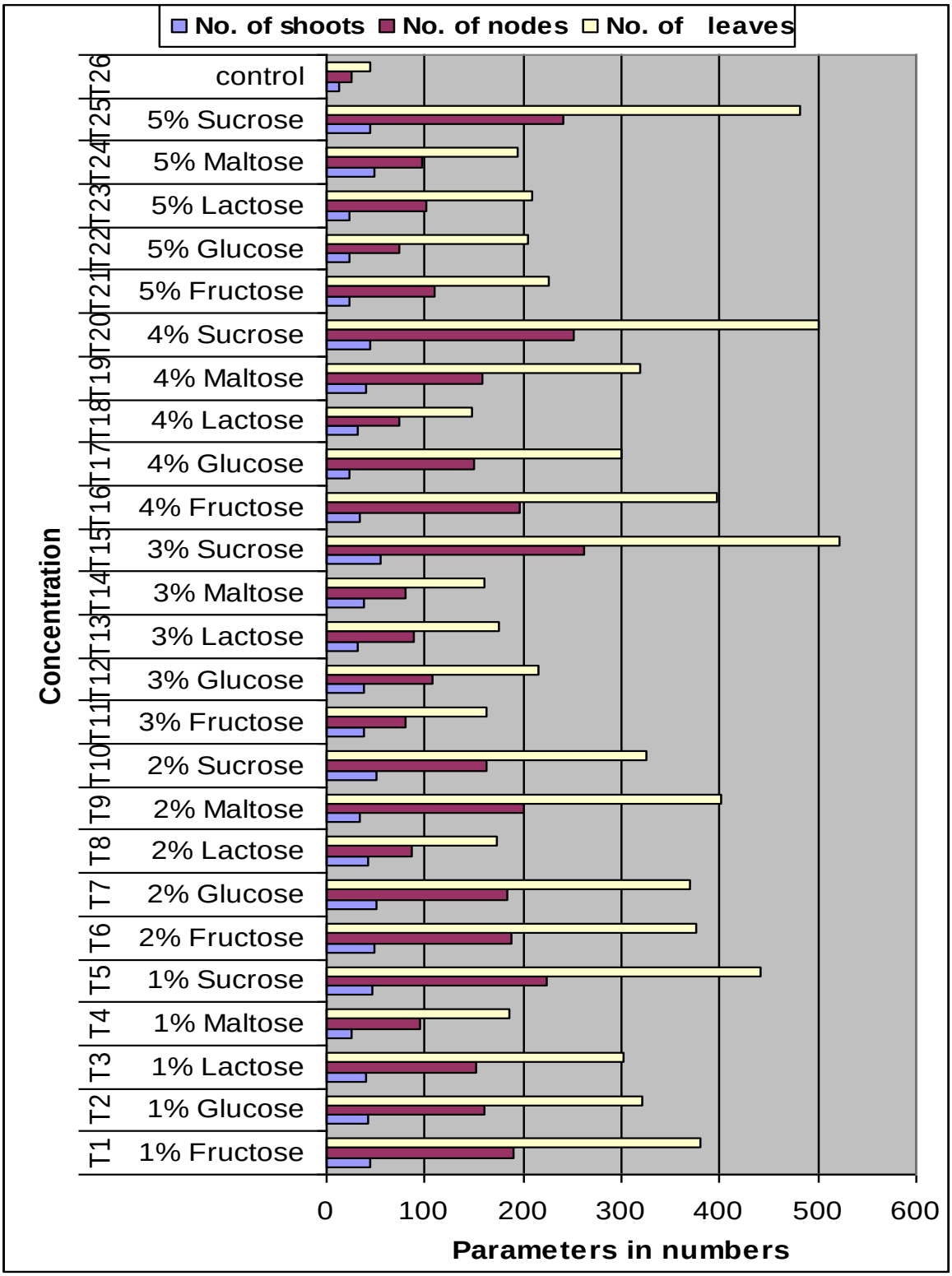


Fig. 11: Influence of various sugars on multiple shoot formation

4.3.12. Optimization of single nodal explant inoculation density:

To determine optimal inoculation density of single node on semisolid media, different numbers of single node (5, 10, 20 and 30) explants were inoculated on 40 ml of media. Results were shown in the Table No: 6.

Multiple shoot formation was maximum at an inoculation density of 20 nodes per 40 ml, whereas, minimum number of shoots was obtained when 30 nodal explants were inoculated on 40 ml (Treat. No. 4) of semisolid media with slightly abnormal growth. However, Eun Joo and Youup (2005) reported highest number of shoots when higher amount of media (12.5 ml) per explant was used.

Highest number of nodes as well as leaves was observed on an inoculation density of one explant / 2 ml media (20 explants per 40 ml) indicating that it was the best among all tested treatments for overall shoot multiplication. All parameters of shoot vigour changed drastically when inoculation densities were above 20 explant/40 ml media (Table No.6)The leaf colour changed from green to light-green or yellow when an inoculation density of 40 was used which was probably caused by aeration with pure air instead of CO_2 enriched air (Hohe *et al.*, 2001).

Same results with lower requirement of medium per explant was also reported by Adelbearg (2004) who observed that the 5.5 ml of media per explant was optimum for multiplication of *Alocasia microrahiza* on liquid media whereas, on semisolid media, highest multiplication was obtained on slightly higher ratio (6.06 ml /explant).

Table 6: Effect of inoculation density on shoot, nodes and number of leaves

INOCULATION DENSITY				
Treatments		No. of nodes	No. of shoots	No. of leaves
T1	5 explants/40 ml medium	76.00	15.49	152.91
T2	10 explants/40 ml medium	76.92	15.39	156.14
T3	20 explants/40 ml medium	80.72	16.60	160.78
T4	30 explants/40 ml medium	46.15	10.62	92.20
SEm		0.68	0.14	0.46
SEd		0.96	0.19	0.65
CD		2.00	0.40	1.35
CV%		2.38	2.30	0.80

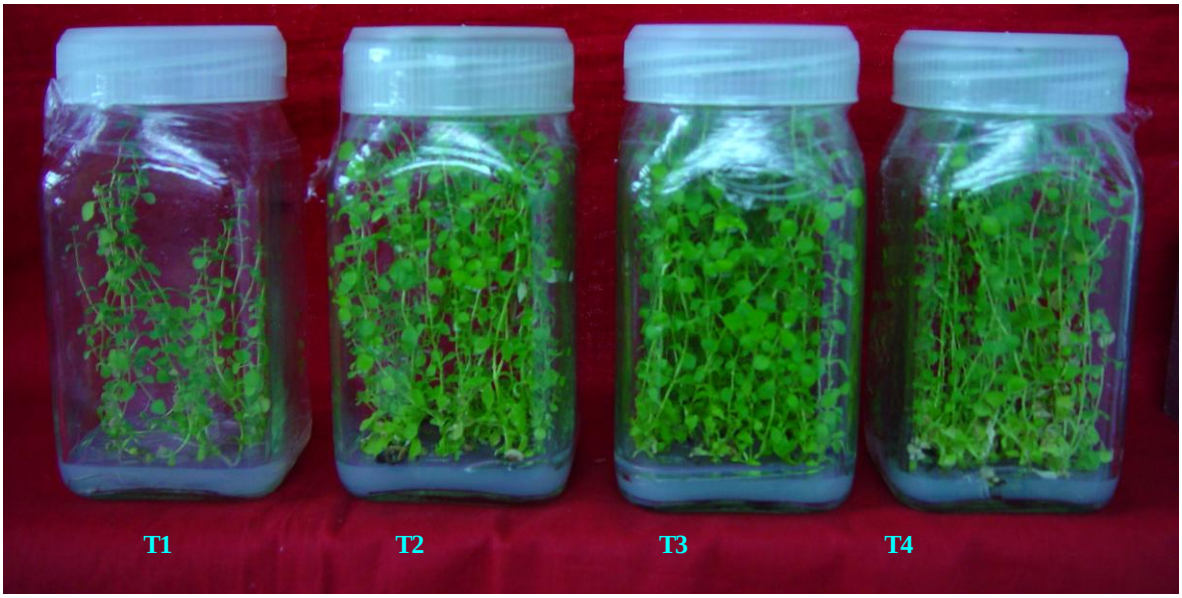


Plate 12: Optimization of single nodal explant inoculation density

T1 **5 explants/40 ml medium**

T2 **10 explants/40 ml medium**

T3 **20 explants/40 ml medium**

T4 **30 explants/40 ml medium**

4.4. Rooting Phase

4.4.1. Effect of nutrient medium concentration on rooting:

Various concentrations of MS medium (1/4, 1/3, 1/2 and full strength) were used to investigate their effect on rooting of stevia shoots. Results showed excellent rooting percentage (100%) in all the treatments tried (3.9.2) secondary root formation was observed at lower concentrations of (1/4 and 1/3 strength) MS medium. However, highest number of roots per shoot was obtained in half strength MS medium. 1/3 strength of MS medium was found better for root initiation (3 days), average length (5.25 cm) and longest root (8.22 cm)(Fig. 12). These results are in agreement with previous reports on stevia rooting (Yang and Chang, 1979; Constantine and Cachita-cosma, 1997) wherein, satisfactory rooting was observed on full strength MS nutrient medium. However, it has been also observed that the transfer of rooted shoots from high strength medium to less concentrated one enhanced rooting (Filho *et al.*, 1992; Jaime and De silva, 2003). Bondarev *et al.* (2003) found better elongation of root on 1/3 length of MS medium and reported that the lower concentration of MS salts was better for increasing the number of roots. Here the favorable effect of a diluted mineral solution on rooting can be explained by the reduction of nitrogen and phosphate concentration in the medium (Driver and Suttle, 1987).

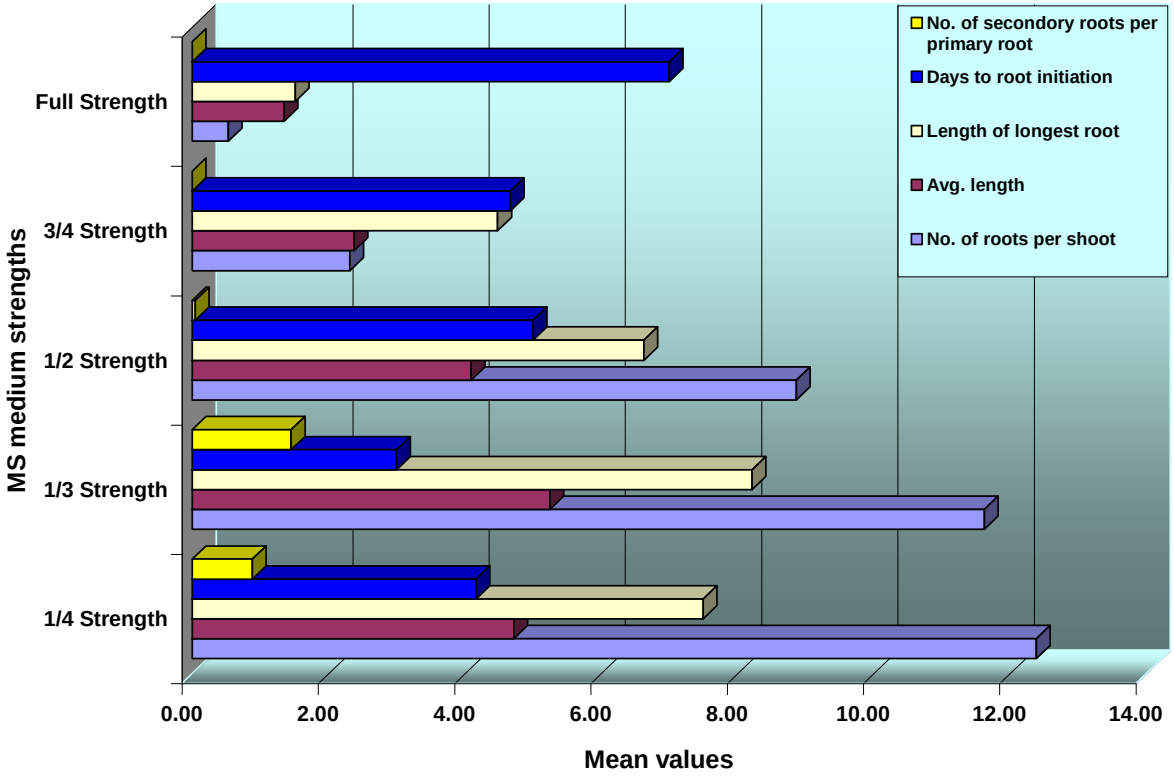


Fig. 12: Effect of concentration of the nutrient medium on rooting

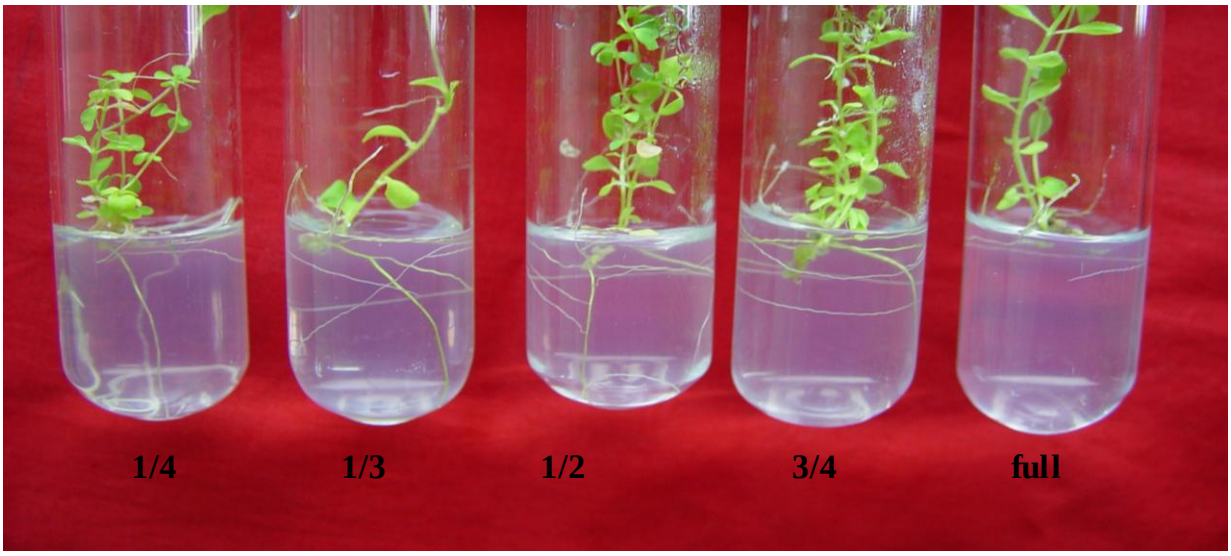


Plate 13: Effect of concentration of the nutrient medium on rooting (21 days after inoculation)

4.4.2. Effect of different PGRs on rooting

The time taken for root initiation, number of roots, length of roots and presence of callus were significantly influenced by different concentrations and types of auxin (IAA, IBA and NAA) used (Fig. 13). Of all three PGRs at different concentrations (0.5 mgl^{-1}, 1.0 mgl^{-1} and 2.0 mgl^{-1}) tested for rooting, the best response (11 roots) was obtained with 1.0 mgl^{-1} NAA followed by 1.0 mgl^{-1} IBA and 1.0 mgl^{-1} IAA. Earlier, Swanson *et al.* (1992) also reported that 1.0 mgl^{-1} NAA being the best supplement on media for rooting in stevia whereas, Tamura *et al.* (1988) observed better rooting on MS medium supplemented with 0.5 mgl^{-1} IBA. In the present experiment regarding the effects of IBA, at 0.5 mgl^{-1} induced less root formation (4 roots) than intermediate concentration 1.0 mgl^{-1} which recorded maximum roots (8.35) while, at higher level of 2.0 mgl^{-1} recorded the least rooting (3 roots). This is also contradictory to the reports by Erdag and Emek (2005).

The increase (2.0 mgl^{-1}) or decrease (0.5 mgl^{-1}) in concentration from 1.0 mgl^{-1} of NAA or IAA resulted in drastic decrease in mean number of roots per shoot (from 11.08 to 2.4 roots). Fotopoulos and Sotiropoulous (2007) observed increase in mean root number by increasing auxin concentration whereas, NAA and IAA at higher concentration (2.0 mgl^{-1}) proved better for root initiation in Gladiolus cv. Peach Blossom (Priyakumari and Sheela, 2005).

The longest root (6.8 cm) and the maximum average length of roots (4 cm) were observed with treatment devoid of PGRs. Among PGR based treatments, the higher mean length of roots (3.6 cm) and longest root (6.5 cm) obtained with 1.0 mgl^{-1} IAA in comparison with treatment of IBA and NAA. It must be pointed out that although auxins induce shorter roots as compared to the control, the total length of root system did not undergo substantial decrease. This is in complete agreement with the findings of Ferreira and Handro (1988).

The occurrence of callus formation was also not observed in treatment without PGRs (control) or very low (0.0 to 0.7) in medium supplemented with IAA whereas, in other cases (IBA and NAA supplementation) callus formation was very pronounced and

the number ranged from 6 to 6.9 which is comparable to the reports of Constantine and Cachita cosma (1997) and Yang and Chang (1979).

Eventhough, all the treatments responded well in inducing the roots (Plate No. 14), the days to root initiation vary considerably from 3 days in case of 1.0 mgl^{-1} IBA to 7 days growth regulator free control. IBA has been found effective for rooting in other members of Asteraceae (Ault, 2002). Interestingly, the days to root initiation was rather consistent for different concentrations of particular auxin and highest difference of only 2 days was recorded in case of 0.5 mgl^{-1} NAA and 2.0 mgl^{-1} NAA. This type of result may be related to the previous culture supplementations and conditions as observed by Morini *et al.* (2003).

The better response of NAA in root formation may be due to its higher effectiveness than IAA, apparently because it is not destroyed by IAA oxidase or other enzymes and therefore persists longer while, IBA might be rapidly metabolized to IBA aspartate and other conjugates with peptides (Salisbury and Ross, 1992) action which might have been affected by endogenous level of auxins (Chandra *et al.*, 1999). This could be the probable reason behind the observed response of IBA.

The inhibition of root initiation and root elongation by higher concentration (2.0 mgl^{-1}) of auxin may be attributed to ethylene production (Klee and Estelle, 1991) and influence of auxin on the decreased uptake of macronutrients and water uptake from the culture medium (Pushpita *et al.*, 1997).

The formation of callus instead of root initiation on supplementation of higher concentration of IBA or NAA (2.0 mgl^{-1}) could be related to their synthetic nature or supplementation at higher amount than required and accumulation just above the wound site because of polar transport (Taiz and Zeiger, 1991) or due to the presence of light which is considered to involve growth inhibitors produced by root tip/cap (Golaz and Pilet, 1995).

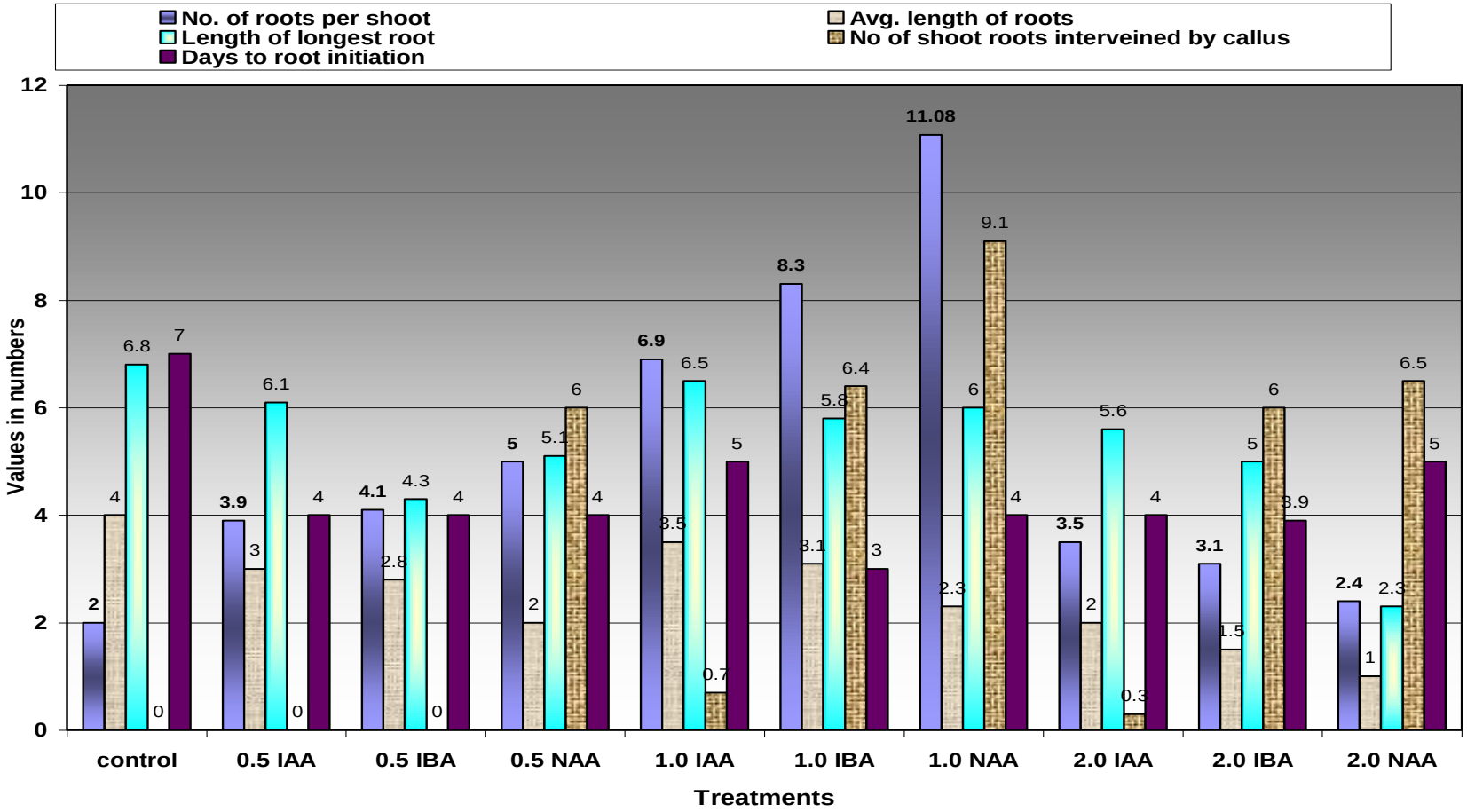


Fig. 13: Effect of different plant growth regulators on rooting

Plate 14: Effect of different PGRs on rooting (14 days after of inoculation)

4.4.3. Effect of combination of auxin on rooting:

To find out the possibilities for improvement of rooting of stevia, further trials were conducted by incorporating three different combinations of auxin (i) IAA + IBA (ii) IAA + NAA and (iii) IBA + NAA using MS medium along with control consisting of the best PGR (IAA) identified from the previous experiment. The results obtained are presented in Fig. No. 14.

The highest number of rooted shoots (11) were recorded on MS supplemented with 0.5 mgl^{-1} IAA + 0.5 mgl^{-1} NAA followed by 9 roots/shoot on IAA + IBA and 7 roots/shoot on control.

The lowest number of roots per shoot (5) was observed when combination of IBA and NAA was used. However, least days (2) were recorded for root initiation in the same treatment (IBA + NAA) while, in other treatments no difference for root initiation was observed (Plate15). The highest occurrence of unwanted callus (6 times) was also observed on IBA + NAA combination as against, least or no callus (1.0 to 0) on IAA + IBA and control (IAA alone). The average length of roots ranged from a minimum of 2.0 cm (IBA + NAA) to highest of 4.35 (IAA + IBA) whereas, the longest root (6.7 cm) was recorded in media supplemented with IAA + NAA and resulted in moderate number of shoots (2) showing callus at the base. The GRs i.e. IAA + IBA resulted in an average root length of 6.5 cm just next to the response obtained with control (6.4 cm).

Saravanan and Nadarajan (2005) obtained the least response to rooting in *Gloriosa superba* L. using 0.5 mg^{-1} IAA. However, better effect of the combination of IBA and IAA was reported by Hassan and Roy (2006). The better performance of IAA + NAA for root formation might be the result of their synergetic interaction.

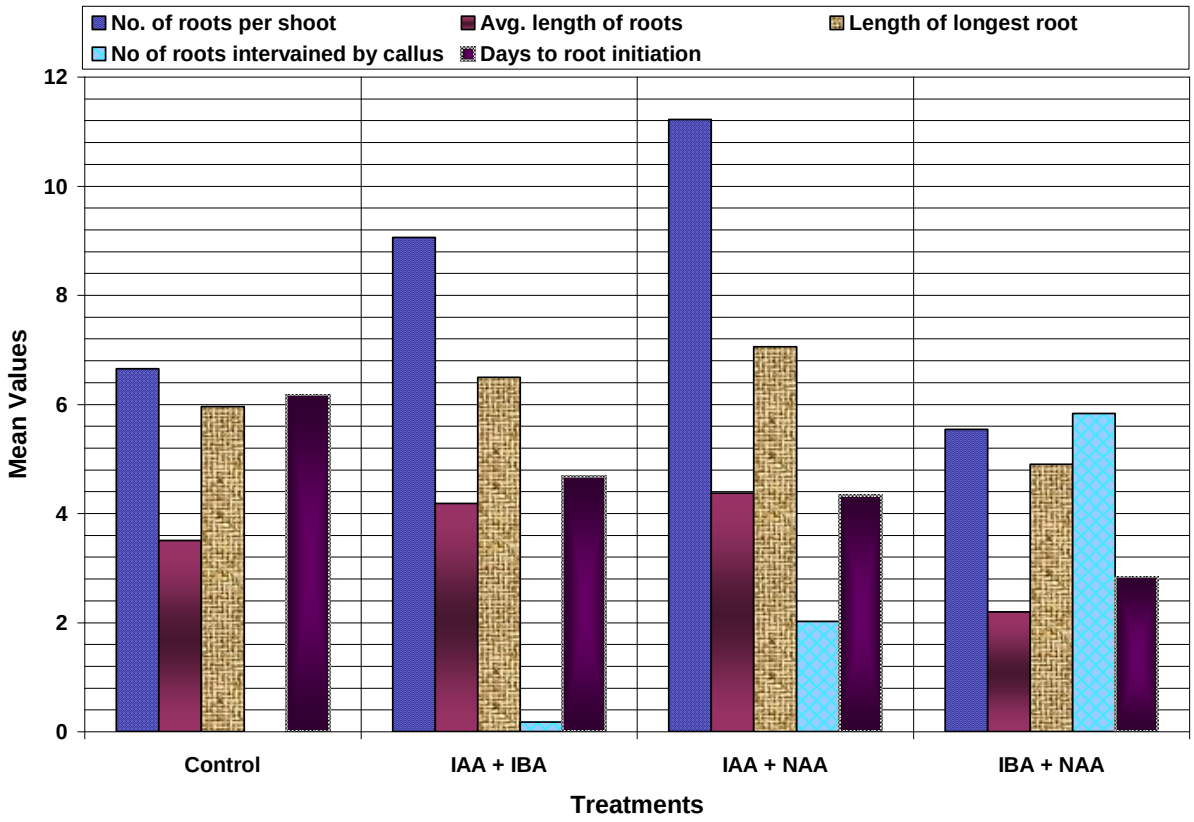


Fig. 14: Effect of different combinations of auxin on rooting

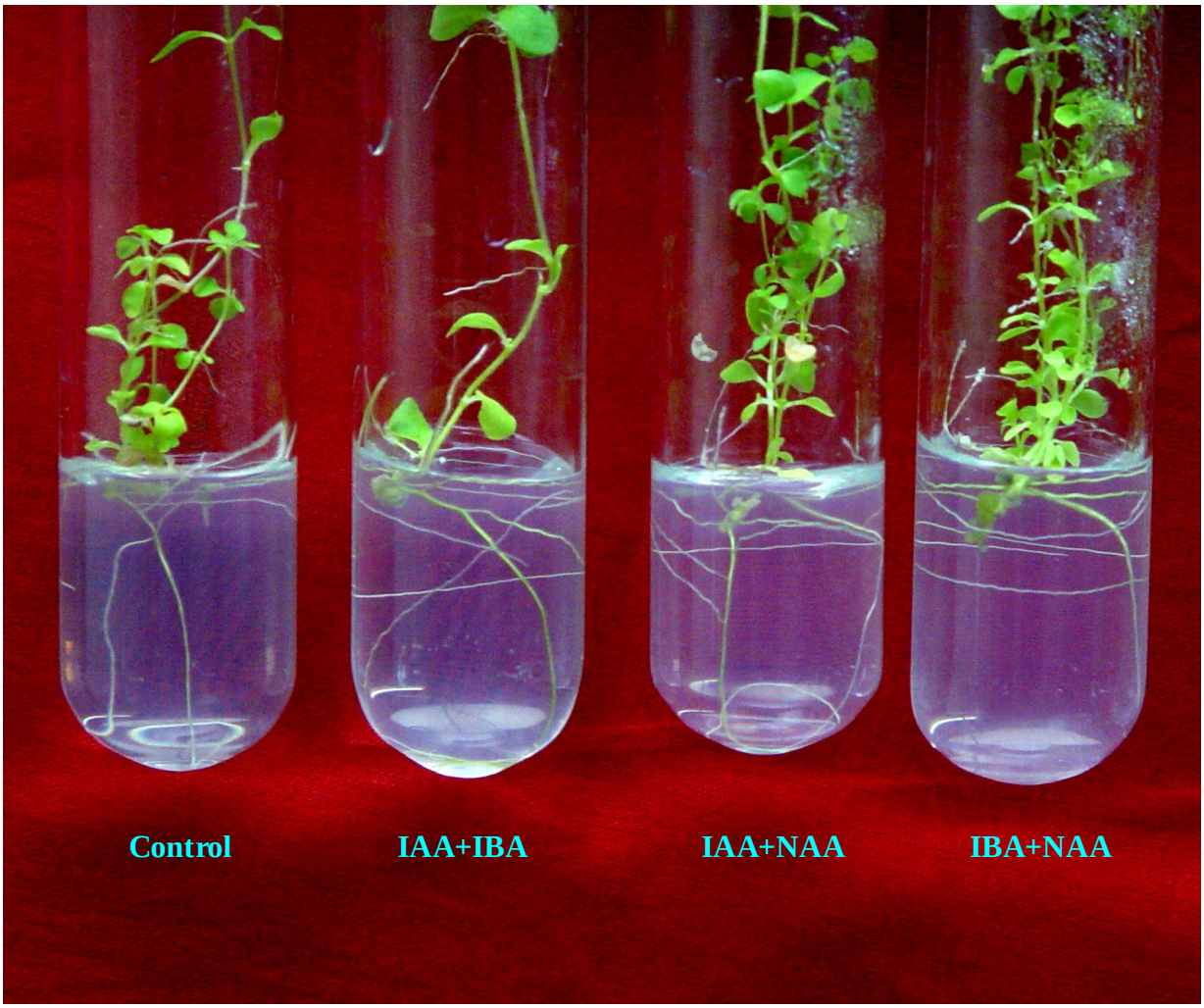


Plate 15: Effect of different combinations of auxin on rooting (14 days after inoculation)

4.4.4. Effect of number of nodes on rooting:

Shoots having different number of nodes (1 node, 2 nodes, 3 nodes, 4 nodes, 5 nodes and 6 nodes) were inoculated on root induction medium to evaluate their effect on root growth and development.

Results showed that the shoots having fewer nodes (2 to 3 nodes) took more days (3.4 to 3.5 days) to initiate roots and the other parameters like number of roots per shoot, average length of roots showed gradual decreasing trend as the number of nodes decreased (Plate 16). However, cent percent rooting on shoots having 5 or 6 nodes and low percentage (48 % to 60 %) was recorded in shoots with single or double nodes. It seems that the total number of nodes present in shoots directly influence the rooting. The present experiment clearly showed that the presence of more nodes (5 nodes) in the explant resulted in more number of roots and length of roots. Moreover, higher number of nodes (4 nodes) bearing explants took less time to initiate roots. Ferreira and Handro (1988) and Tadhani (2006) emphasized the need for longer shoots (4 to 6 cm) having atleast 3 to 4 nodes or more for better rooting in stevia and stated that the rooting process is affected by the presence of nodes; whereas, Pal *et al.* (2005) reported better growth and development of roots from shoots having more number of nodes as compared to shoots with single or lesser number of nodes.

The probable reason for stimulation of rooting by more number of nodes might be related to the age factor and balanced synthesis and translocation of PGRs and other metabolites.

Plate 16: Effect of the number of nodes on rooting (14 days after inoculation)

4.4.5. Effect of activated charcoal on *in vitro* rooting:

Eventhough the beneficial effects of activated charcoal (AC) on rooting in various crops are available in literature, no report on the use of activated charcoal in the rooting process of stevia has been available. Therefore, an experiment was conducted based on eight treatments containing different auxin along with / without activated charcoal to improve the rooting response of stevia shoots and to compare their effect on rooting. The details of the treatments are given in materials and methods (3.9.5) whereas the results are presented in Figure No.12. Highest number of roots per shoot (10.8) was observed in treatment containing NAA and activated charcoal. Treatment without AC (Treat. No. 8) also resulted in comparable number of roots (10.6) whereas, the lowest number of roots per shoot was recorded in IAA based medium with (6.9) or without AC (6.8).

Moreover, the number of roots per shoot was found comparable in the treatments with AC (Treat. No. 2, 4, 6, 8) and without AC (Treat. No. 1, 3, 5, 7). The same tendency was observed in the case of days to root initiation, indicating that AC exerted no significant effect on these parameters. However, significant increase in length of roots was observed in all the treatments augmented with AC (Treat. No. 2, 4, 6, 8) as compared to treatments without AC. The positive effect of AC treatment on elongation of roots may be related to

the faster metabolism of endogenous auxins in the dark surroundings (Maynard and Bassak, 1987).

Decrease in the intervening callus formation at the base of the shoot where the root initiate, was also noted in treatments with AC regardless of the type of PGRs used; whereas, maximum decrease in callus formation (70 % to 10%) was seen in treatments supplemented with NAA and AC followed by IBA based treatments and IAA fortified media. The beneficial effect of AC may include adsorption of components of medium, agar impurities, and inhibitory growth byproducts, media darkening, pH stabilization and catalyzed breakdown of sucrose during sterilization with subsequent absorption of hydroxyl methyl furfural. However, interaction is likely and could be more important than isolated effect (Winkle and Pullman, 1995).

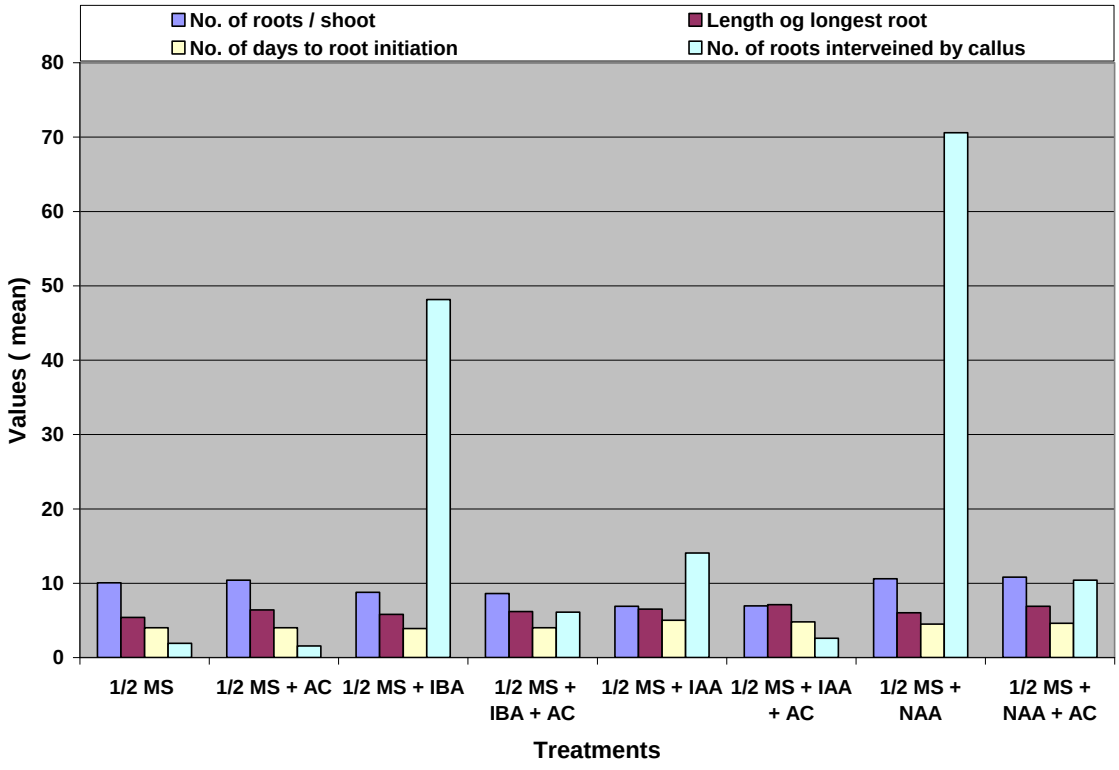


Fig No. 15: Effect of activated charcoal on *in vitro* rooting

Plate 17: Effect of activated charcoal on *in vitro* rooting

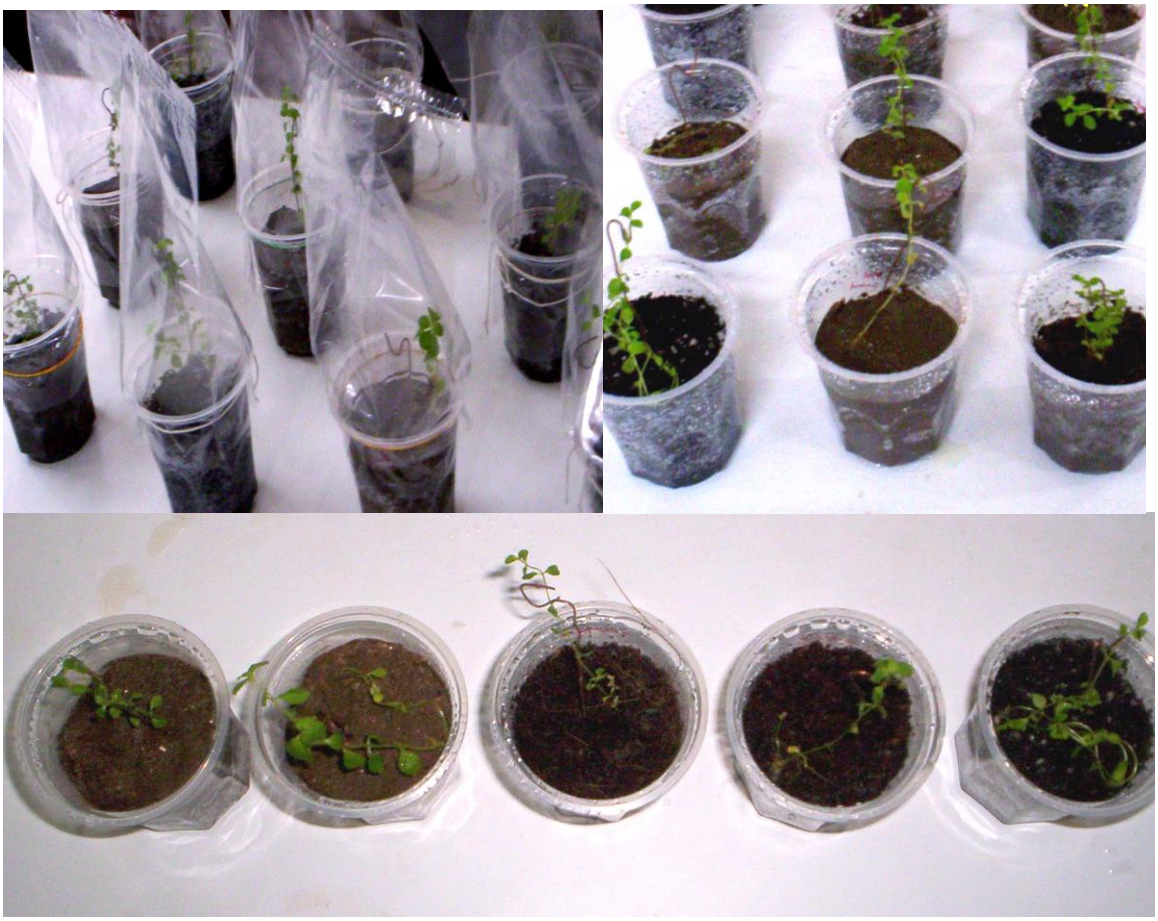


Plate 18: Primary hardening of stevia plantlets (from lab to net house)

4.5. Acclimatization stage:

4.5.1. Effect of different potting mixtures on survival of plantlets during acclimatization:

Well rooted and healthy plantlets with approximately 3-5 nodes and minimum of four roots were transferred in pots containing different potting mixtures. Twenty six types of various potting mixtures were tried consisting of cocopeat, farm yard manure, sand, soil and vermiculite. The results obtained are presented in Table No.7.

The plantlets transferred on potting mixture containing FYM showed lower survival percentage (from 0 to 15 %) while, higher number of survived plants was observed (45 to 75) in potting mixture consisting of cocopeat alone or in combination with other substrates. Overall, the highest number of survived plant (54) was obtained with cocopeat alone while, least number of survivals was observed on FYM, when used alone.

Number of survived plants on potting mixtures containing two substrates ranged from 9.83 to 72.18 in FYM : sand and cocopeat : sand mixture, respectively. Minimum mean number of survived plants was 14.3 on FYM : sand : soil while highest number of survived plantlets (70.83) was observed on cocopeat : sand : soil mixture. In potting mixture consisting of four different substrates resulted in significantly higher number of survival plants. In lowest number of survival of plants (16.02) was recorded in case of cocopeat : FYM : sand : vermiculite whereas, highest number of survived plantlets (75 %) was observed in cocopeat : sand : soil : vermiculite. However, when FYM was added in this potting mixture, the mean number of plants survived was drastically decreased to 15.85. This indicates the incompatibility problem or harmful effect of FYM on stevia plants during acclimatization. However, the total number of survived plants were increased with the increase in number of substrates used in potting mixture compared to potting mixtures consisting of less number of substrates indicating that the potting mixture of different substrates are better than single substrates.

Contrary to the present results, Mathew (1995) reported the maximum number of survived plants potting mixture containing sand : soil : FYM(1:1:1). Thus, the potting mixture (T24) proved best during acclimatization. These results are also contradictory to

the findings of Benerjee *et al.* (2004), who used soil: sand : FYM (3:1:1) as potting mixture during initial hardening of *Cinaria muritima* L. However, the present findings are in agreement with the reports of Chandra *et al.* (2003) and Tadhani *et al.* (2006) who reported the use of cocopeat and sand for acclimatization of herbaceous plants.

The plantlets transferred on potting mixture containing FYM showed lower survival index (from 0 to 15) while, higher number of survived plants was observed (45 to 75) in potting mixture consisting of cocopeat alone or in combination with other substrates.

The low *in vivo* survival may be related to the alteration in development and physiology of *in vitro* raised plants leading to low photosynthetic capacity and deficient water uptake and poor conducting system. Slow growth or low survival under *ex vitro* condition is known to be related with low epicuticular wax and defective stomatal functioning and higher water loss (Huylenbroeck *et al.*, 2000).

However, better survival of plantlets found in the present experiment on cocopeat based substrates might be due to its better water holding capacity and good aeration to the young root system (Chandra *et al.*, 2003).

Table 7: Analysis of variance table for effect of different potting substrates on survival percentage of plantlets during acclimatization

ANOVA						
SOURCE	**DF**	**SS**	**MS**	**CAL F**	**TAB F**	**RESULT**
TRET	25	71952.86	2878.11	614.62	**1.591**	*
ERR	130	608.75	4.68			
TOT	155	72561.61				
SEm		0.88				
SEd		1.24				
CD		2.47				
CV%		**6.13**				

Field planting:

General observation was made to certain the survivability of *in vitro* raised plants in field. The plants were found growing healthy even after three months period (Plate 20).

Table 8: Effect of different potting substrates on survival percentage of plantlets during acclimatization

Treatment code	Potting mixture	No. of plant survived	Survival index (Range)	Total number of plants survived
T1	Cocopeat alone	54.74	0 to 54	162.35
T2	Farmyard manure (FYM) alone	0.00		
T3	Sand alone	50.48		
T4	Soil alone	38.46		
T5	Vermiculite alone	18.67		
T6	Cocopeat: Sand	72.18	9 to 72	329.84
T7	Cocopeat: Soil	52.19		
T8	Cocopeat: FYM	25.67		
T9	Cocopeat :Vermiculite	45.34		
T10	FYM :Sand	11.11		
T11	FYM: Soil	10.15		
T12	FYM :Vermiculite	9.83		
T13	Sand: Soil	35.12		
T14	Sand: Vermiculite	37.83		
T15	Soil: Vermiculite	30.42		
T16	Cocopeat :FYM: Sand	36.74	14 to 70	287.77
T17	Cocopeat :FYM: Vermiculite	45.50		
T18	Cocopeat : Soil : Vermiculite	67.01		
T19	Cocopeat : Sand: Soil	70.83		
T20	FYM : Sand : Soil	16.02		
T21	Sand : Soil: Vermiculite	51.67		
T22	Sand: Soil : FYM : Vermiculite	14.50	16 to 75	121.42
T23	Cocopeat :FYM : Sand : Soil	17.53		
T24	Cocopeat : Sand : Soil : Vermiculite	75.06		
T25	Cocopeat: FYM : Sand: Vermiculite	14.33		
T26	Cocopeat: FYM : Sand : Soil: Vermiculite	15.85	15	15.85

Plate 19: Fully hardened stevia plants under net house condition

Plate 20: Normal growth of in *vitro* raised stevia plants under field condition

SUMMARY AND CONCLUSION

Surface sterilization

Various concentrations of $HgCl_2$ for different duration were tried to investigate its effectiveness in controlling surface contamination with satisfactory survival of explants. The results showed least contamination by treating explants with 0.5 % $HgCl_2$ for 20 min with poor survival (less then 50 %). Therefore, four different antibiotics *viz.* kanamycin, streptomycin, cefotaxime and tetracycline were tried as surface sterilant for total elimination of contamination from explants. The percentage survival was higher when kanamycin was used which was followed by cefotaxime. For further improvement of this procedure, six different combinations of antibiotics/ bacteriocides and antifungal agents were used sequentially to eliminate surface contaminants completely. Lower concentration of bavistin followed by kanamycin and $HgCl_2$ sequentially resulted in complete elimination (more then 90 %) of surface contaminants and satisfactory survival percentage (more then 70 %) of the explants.

Culture establishment stage

For culture establishment, five treatments including half strength MS medium, full strength MS, half strength B_5 and full strength B_5 medium were tested. Results showed that the percentage establishment and growth of cultures on full strength MS medium was better than other media tried while, full strength B_5 medium was found better for bud sprouting.

Various treatments of light and dark regimes for a period of 14 days after initial inoculation were also tried to enhance the culture establishment. Best establishment (98.36) percentage was recorded in treatment consisting of 4 days of complete dark followed by 3 days of continuous light regime followed by the same treatments repeatedly upto 14 days.

Multiplication stage

For induction of multiple shoot formation and further development of shoots, MS medium supplemented with four different cytokinins *viz.* BA, Kinetin, Thiadiazuron and Zeatin, at various levels. The number of shoots regenerated varied significantly at different levels of various cytokinins. Highest degree of shoot

proliferation was recorded on medium containing 2.0 mgl^{-1} BA characterized by maximum number of shoots, nodes and leaves.

After comparing various cytokinins at different concentrations, trials were conducted for further improvement in multiple shoot formation and development by using various combinations of two cytokinins viz., BA and Kn. Significant differences were observed among different treatments tried. The maximum number of shoots was observed with the lowest level of BA and Kn. However, the combination of cytokinins failed to further improve multiple shoot formation and development as compared to the previous experiment.

To find out the best medium for multiple shoot formation and development response of *in vitro* cultures, five different media (MS, B_5, LS, N & N and White's) at full strength were examined. MS, B_5 and LS media were found worth experimenting and therefore in next experiment, total six treatments consisting of three types of media B_5, LS and MS media at full and half strength were evaluated for identifying the best medium among them for increased multiplication and development of stevia shoots. Results showed that the MS medium performed better than other media and an experiment was carried out to optimize its concentration for further increasing multiplication potential of shoots. Half strength, 3/4 strength, 1/1 (full) strength and 1½ (1.5) strength of MS medium were examined to find out its optimum concentration for shoot multiplication and development. Cultures grown on ¾ strength medium attained the highest average length (4.7 cm). The same treatment also resulted in the highest number of shoots (8.1), maximum number of nodes (38.2) and leaves (80.5).

The effect of initial position of explants on its mother plant before isolation was also found to influence its morphogenic expression under *in vitro* conditions. Among different explants tried, shoot initials isolated from the terminal/upper most position showed overall better multiplication and development with the highest number of shoots (49.3), more number of nodes (282.3) and higher number of leaves (562) along with higher length of shoots (4.68 cm)

Further experiments were setup to find out the effect of retention of nodes on shoot formation and multiplication and a comparison was made using five types of explants differing in number of nodes viz. single node, shoot with 2 nodes, shoot with 3 nodes, shoot with 4 nodes and shoot with 5 nodes. The total number of shoots, nodes and leaves were increased as the number of nodes was increased in the explant. The explant consisted of single node gave rise to the lowest number of shoots (4.5), nodes (2.3) and leaves (4.8).

Effect of different culture environments on multiplication was evaluated through trials by examining the performance of *in vitro* grown shoots under three different light intensity regimes including low light intensity (20 µmol m^{-2} s^{-1}), medium light intensity (40 µmol m^{-2} s^{-1}) and high light intensity (60 µmol m^{-2} s^{-1}). Results showed that the explants grown under medium light intensity(40 µmol m^{-2} s^{-1}) resulted in higher multiplication (19.23) of shoots and highest number of nodes and leaves and the data were significant. Similar trend was observed for the days to bud sprouting in this study wherein minimum period of two days was required for sprouting as observed under medium light intensity.

Experiments were also performed to find out photomorphogenic effects of various light/dark regimes. Different photoperiods including 0 hrs. light /24hrs. dark, 24 hrs. light / 0 hrs. dark ,16 hrs. light / 8 hrs. dark, 8 hrs. light / 16 hrs. dark and 12 hrs. light /12hrs. dark periods were tried. Maximum number of multiple shoots was observed when the explants were grown under 8 hrs. light/ 16 hrs. dark period.

To find out relationship between subculture passage and the morphogenic potential of plantlets, an experiment was carried out to assess the effect of number of subculture on multiplication. Higher number of shoots (6.03) was obtained on medium supplemented with 0.5 mgl^{-1}BA after sixth subculture as compared to shoots obtained on medium supplemented with 2.0 mgl^{-1} BA.

For standardization of sugar concentration and evaluate their influence on shoot multiplication, five types of sugars i.e. Fructose, Glucose, Lactose, Maltose and Sucrose at various concentrations (1 % to 5 %) were compared at various levels. Among different treatments tried, 3% sucrose resulted in significantly higher number

of shoots while, the lowest number of shoots was observed on media supplemented with 5 % lactose.

To standardize optimal inoculation density of single node on semisolid media, different numbers of single node (5, 10, 20 and 30) explants were inoculated on 40 ml of media. Maximum multiple shoot formation was recorded at an inoculation density of 20 nodes per 40 ml, whereas, minimum number of shoots (10.62) was obtained when 30 nodal explants were inoculated on 40 ml of semisolid media with slightly abnormal growth.

Rooting Stage

Various concentrations (1/4, 1/3, 1/2 and full strength) of MS medium were used to investigate their effect on rooting of stevia shoots. Results showed excellent rooting percentage in all the treatments tried. However, highest number of roots per shoot was obtained in half strength MS medium. 1/3 strength of MS medium was found better for root initiation, average length and longest root.

The time taken for root initiation, number of roots, length of roots and presence of callus were all significantly influenced by different concentrations and types of auxin (IAA, IBA and NAA) used. Of all three PGRs at different concentrations tested for rooting (0.5 mgl^{-1}, 1.0 mgl^{-1} and 2.0 mgl^{-1}), the best response was obtained with 1.0 mgl^{-1} NAA followed by 1.0 mgl^{-1} IBA and 1.0 mgl^{-1} IAA.

Further trials were conducted by incorporating three different combinations of auxin IAA + IBA , IAA + NAA and IBA + NAA using MS medium along with control consisting of the best PGR (IAA) identified from the previous experiment. The highest number of roots was recorded on MS medium supplemented with 0.5 mgl^{-1} IAA + 0.5 mgl^{-1} NAA.

Physiological attributes of nodes were also investigated by using shoots having different number of nodes (1 node, 2 nodes, 3 nodes, 4 nodes, 5 nodes and 6 nodes) for root induction and development. Results showed that the shoots having fewer nodes (2 to 3 nodes) took more days (3 to 7 days) to initiate roots and the other

parameters like number of roots per shoot, average length of roots which showed gradual decreasing trend as the number of nodes decreased.

To improve the rooting response of stevia, an experiment was conducted comprising of eight treatments involving different auxins along with / without activated charcoal. Significant increase in length of roots was observed in all the treatments augmented with AC (Treat. No. 2, 4, 6, 8) as compared to treatments without AC. Decrease in the intervening callus at the base of the shoot where the root initiate, was also noted in treatments with AC regardless of the type of PGRs used. Highest number of roots per shoot was observed in treatment containing NAA and activated charcoal.

Acclimatization/Hardening stage

Well rooted and healthy plantlets with approximately 3-5 nodes and minimum of four roots were transferred in pots containing different potting mixtures. Twenty six types of potting mixtures were tried consisting of cocopeat, farm yard manure, sand, soil and vermiculite.

The plantlets transferred on potting mixture containing FYM showed lower survival percentage while, higher number of survived plants (<60) was observed in potting mixture consisting of cocopeat alone or in combination with other substrates. Overall, the highest number (index:75) of survived plant was obtained with cocopeat alone while, least number of survivals was observed on FYM, when used alone.

CONCLUSION

1) Sequential surface sterilization with 200ppm bavistin, 200ppm kanamycin and 0.1% $HgCl_2$ (5 minutes) was found to effective for obtaining contamination free cultures
2) Full strength MS medium supplemented with 0.2 mgl^{-1} BA and 0.2 mgl^{-1} NAA concentration was resulted in initiation or establishment of Stevia cultures
3) Establishment of Stevia cultures was not much affected by various light and dark cycle regimes
4) Three fourth strength of MS medium supplemented with 2.0 mgl^{-1} BA was optimum for maximum multiplication of shoots
5) Shoots having three nodes were found to produce more number of shoots (7.8) and hence better as explant for multiplication stage
6) Medium level of light intensity (40 μmol m^{-2} s^{-1}) was required for better multiplication when maintain under 8 hrs light and 16 hrs dark photoperiod
7) Three percent sucrose was optimum for better multiplication of shoots
8) Inoculation density of 120 nodes per 140 ml medium can be used for better growth and development of shoots
9) Half strength MS medium supplemented with 0.5 mgl^{-1} IAA and 0.5 mgl^{-1} NAA along with 2 % sucrose was found optimum for root initiation and development
10) Shoots having 5 nodes can be used for early (3 days) days and better root initiation
11) Potting mixture of cocopeat and sand (1:1) was best suited for overall development of plants during primary hardening
12) There was no variability observed in the tissue culture raised plants up to 6^{th} cycle which shows suitability of the protocol for commercialization
13) An efficient and repeatable regeneration protocol of Stevia through *in vitro* propagation has been achieved.

Note : The protocol developed has been given in the Appendix II.

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APPENDIX - I

Composition of different media used:

Chemicals	MS medium	LS medium	B_5 medium	Nitsch and Nitsch medium	Whites medium
	mgl^{-1}				
KNO_3	1900	1900	2528	950	80
NH_4NO_3	1650	1650	-	720	-
$CaCl_2.2H_2O$	440	440	150	166	-
$MgSO_4.7H_2O$	370	370	246	180	750
KH_2PO_4	170	170		68.0	-
Na Fe-EDTA	36.7	36.7	37.2	37.5	-
$ZnSO_4.7H_2O$	8.6	8.6	2.0	1.6	3.0
H_3BO_3	6.2	6.2	3.0	10.0	1.5
KI	8.3	8.3	0.75	0.25	0.75
$Na_2MoO_4.2H_2$	0.25	0.25	0.25	0.025	-
$CuSO_4.5H_2O$	0.025	0.025	0.025		0.01
$CoCl_2.6H_2O$	0.025	0.025	0.025	25.0	-
$MnSO_4.4H_2O$	16.9	16.9	10.0	-	5.0
Myo - Inositol	100.0	100.0	100	100	-
Thiamine	0.1	4.0	10.0	0.5	0.01
Pyridoxine	0.5	-	1.0	0.5	0.01
Nicotinic acid	0.5	-	1.0	5.0	0.05
Glycine	2.0	-	-	2.0	3.0
NaH_2PO_4	-	-	150	-	19
$Ca(NO_3)_2$	-	-	-	-	300
Na_2 So_4	-	-	-	-	200
$Fe_2(SO_4)_3$	-	-	-	-	2.5
Biotin	-	-	-	0.05	-
Folic Acid	-	-	-	0.5	-

APPENDIX - II

PROTOCOL

Explant surface sterilization with 200ppm bavistin, 200ppm kanamycin and 0.1% $HgCl_2$ (5 minutes)

↓

Initiation or establishment of Stevia cultures on full strength MS medium supplemented with 0.2 mgl^{-1} BA and 0.2 mgl^{-1} NAA

↓

Multiplication of shoots having three nodes on three fourth strength of MS medium supplemented with 2.0 mgl^{-1} BA and 3 % sucrose

↓

Maintain under 8 /16 hrs photoperiod and light intensity of 40 µmol m^{-2} s^{-1}

↓

Root initiation and development of shoots on half strength MS medium supplemented with 0.5 mgl^{-1} IAA and 0.5 mgl^{-1} NAA along with 2 % sucrose

↓

Primary hardening using potting mixture of cocopeat and sand (1:1)

↓

Secondary hardening under shed house

↓

Field transfer