EFFECT OF GROWTH REGULATORS ON MICROPROPAGATION OF STEVIA

(*Stevia rebaudiana* Bert.)

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CERTIFICATE

This is to certify that thesis entitled, " Effect of Growth Regulator on Micro Propagation of Stevia (*Stevia rebaudiana* Bert.)" submitted to Faculty of Agriculture, Sher-e-Bangla Agricultural University, Dhaka, in partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE in HORTICULTURE AND POSTHARVEST TECHNOLOGY,** embodies the result of a piece of bona-fide research work carried out by **Md. Hasan Waliullah,** Registration No. 25222/00344 under my supervision and my guidance. No part of the thesis has been submitted for any other degree or diploma.

I further certify that such help or source of information, as has been availed of during the course of this investigation has duly been acknowledged.

Dated:
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DEDICATED
TO MY
BELOVED PARENTS

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ABSTRACT

This study was concerned with the micropropagation of stevia by tissue culture, in order to establish an efficient and reproducible *in vitro* propagation technique. The explants, shoot tip, leaf base with petiole and nodal segment of stevia were cultured on MS medium supplemented with 5 different concentrations (0.0,1.0,2.0,3.0,4.0 and 5.0 mg/L) of BAP for shoot proliferation and elongation of stevia. Nodal segment performed best and BAP 3.0 mg/L was found optimum for shoot proliferation and elongation. The experiment with various cytokinin showed that BAP applied alone was the most effective cytokinin for stevia. When BAP was used in combination with 2ip and KN, the number of shoot and their length were decreased, whereas the highest number of shoot (8.0) and length (7.8 cm) was found with the application of BAP 3.0 mg/L. The best response towards root induction was achieved on ½ MS medium supplemented with 1.5 mg/L NAA. Callus induction was observed, the best on MS medium containing 3.0 mg/L of 2, 4-D. Callus (0.3g) explanted on MS basal medium supplemented with different concentration and combination of NAA and BAP (T_1-control, T_2-1.0 mg/L BAP + 0.5 mg/L NAA, T_3-1.0 mg/L BAP + 1.0 mg/L NAA, T_4- 1.0 mg/L BAP + 2.0 mg/L NAA, T_5-2.0 mg/L BAP + 1.0 mg/L NAA, T_6-2.0 mg/L BAP + 2.0 mg/L NAA) to observe their effect on the subsequent growth and development of initiated stevia plantlets at 30, 60 and 90 days after inoculation (DAI). However, T_2 (1.0 mg/L of BAP + 0.5 mg/L of NAA) gave the best result (8.9 shoots/ explant) in respect of the highest shoot proliferation and the highest number of root formation was 9 roots /shoot under T_3 (1.0 mg/L BAP + 1 mg/L NAA) treatment at 90 days after inoculation (DAI). The regenerated plantlets were successfully established into the pots after proper hardening. The cocodust medium was found the best substrate for *ex vitro* establishment of stevia.

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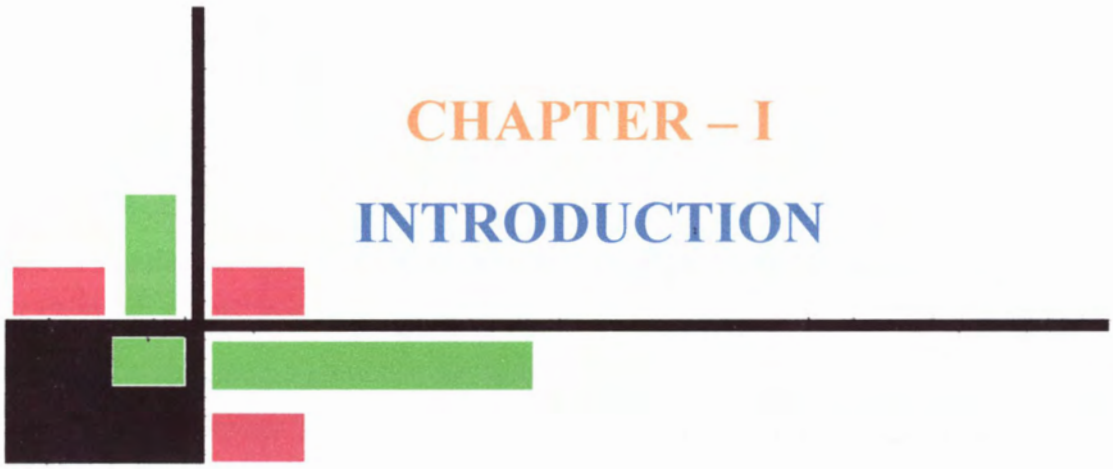
LIST OF ABBREVIATIONS

EXPANSION	ABBREVIATIONS
Bangladesh Agricultural Research Institute	BARI
Bangladesh Sugarcane Research Institure	BSRI
Horticulture Research Centre	HRC
Standard Error	SE
Completely Randomized Design	CRD
Duncan's Multiple Range Test	DMRT
6 – Benzylaminopurine	BAP
Indole - 3 – Butyric Acid	IBA
Murashige and Skoog	MS
Naphthalene -1- Acetic Acid	NAA
Kinetin	Kn
2-Isopentenyladenine	2ip
Thidiazuron {N-Phenyl-N-(1, 2, 3-Thiadiazol-5-yl) Urea	TDZ
Days After Inculation	DAI
Hydrogen ion Conc.	pH
Hydrochloric acid	HCl
For Example	e.g.
Et alii=other people	*et al.*
Et cetera=and others	etc.
id est= in other words	i.e.
Figure	Fig.
Gram	g
Milli gram	mg
Centimeter	cm

CHAPTER – I

INTRODUCTION



CHAPTER I

INTRODUCTION

Stevia (*Stevia rebaudiana* Bert.) is a small, herbaceous, semi-bushy plant belongs to the family Compositae (Plate 1). Stevia originates in the highland regions of Northeastern Paraguay that occur between 23 and 24 south latitude. Plants are perennial in nature and occur on the edges of marshes or in grassland communities on soils with shallow water tables, the soils are typically infertile acid sands or mucks (Shock, 1982). The climate can be considered as semi-humid subtropical with temperatures ranging from 15^0-30^0C. So, the climatic condition of Bangladesh is suitable for stevia growing.

A number of compounds are present in the leaf tissue that has a potential food use as low calorie sweeteners. The leaves of the herb, known to the Guarani Indians of Paraguay as Ka-a He-e, have been used for centuries as a sweetener for bitter drinks such as maté (Soejarto *et al.*, 1983). Eight diterpene glycosides with sweetening properties have been identified in leaf tissues of Stevia. The four major sweeteners are- stevioside, rebaudioside A, rebaudioside C and dulcoside A. Stevioside and steviol are neither mutagenic nor clastogenic *in vitro* at the limited doses (Suttajit *et al.*, 1993). Stevia shows its greatest potential as a natural source alternative to the synthetic sweetening agents that are now available to diet conscious consumers. This is especially relevant in context of the current movement of society towards more natural foods.

The first reports of commercial cultivation in Paraguay were in 1964 (Katayma *et al.*, 1976; Lewis, 1992). Sumida (1968) starts a large effort aimed at establishing Stevia as a crop in Japan. Since then, Stevia has been introduced as a crop in a number of countries including Brazil, Korea, Mexico, United States, Indonesia, China, Tanzania and since 1990 Canada (Lee *et al.*, 1979; Goenadi, 1983; Shock, 1982; Saxena and Ming, 1988; Brandle and Rosa, 1992; Fors, 1995). Currently Stevia production is centered in China and the major market in Japan (Kinghorn and Soejarto, 1985). In Japan alone, an estimated 50 tons of stevioside is used annually with sales valued in the order of $220 million Canadian (Brandle and Rosa, 1992). To date there have been no reports of adverse effects from the use of stevia products by humans

(Kinghorn and Soejarto, 1985). According to World Health Organization (WHO) findings it regulates blood pressure, fights cavities, induces pancreas to produce more insulin and act as bactericidal agent (Bhosle, 2004).

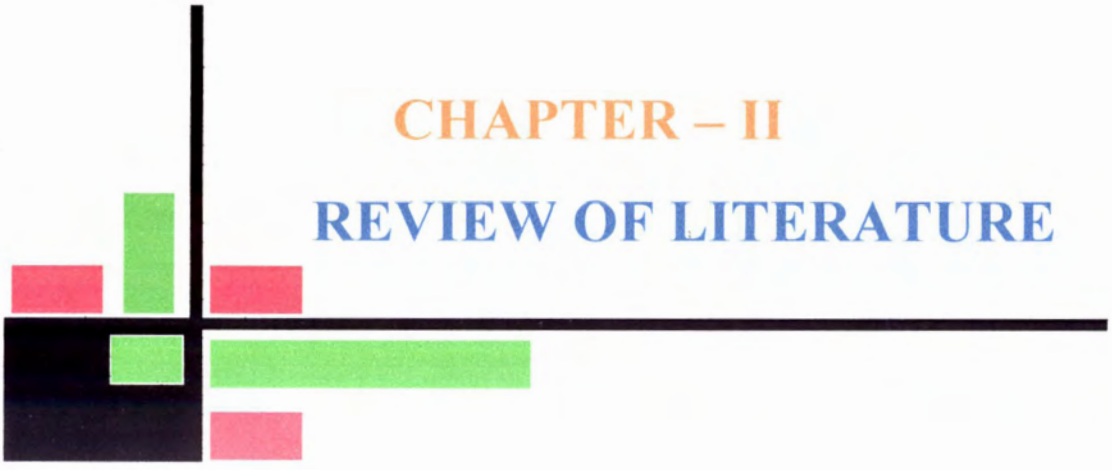
The property of the species that called attention to the plant was the intense sweet taste of the leave and aqueous extracts. The dry leaves of this plant are sweeter 30 times than sugar, with zero calories. Where as pure extract stevioside is non-caloric and 300 times sweeter than sugar (Bhosle, 2004). Stevia is nutrient-rich, containing substantial amounts of protein, phosphorus and other important nutrients (Viana and Metievier, 1980). Stevioside is of special interest to diabetics, persons with hyperglycemia and the diet conscious. A sugar-free, no-calorie natural sweetener is especially helpful for people who are diabetic, prone to yeast infections, or trying to lose a few extra pounds by controlling calories. Additions of Stevia powder or liquid a pinch or drops at a time to tea, coffee, dairy products, or juices sweeten to taste. Some people detect a slight licorice aftertaste, depending on potency.

The propagation and cultivation of this plant deserve due research attention. Seed germination of stevia is often poor. Therefore, there are basically two options for multiplication. The first is the tissue culture and second the stem cutting. The plants are propagated vegetatively by stem cutting, which is a slow process (Miyagawa *et al.*, 1986). Moreover, the cuttings are sometimes infected with diseases. Application of tissue culture technique has therefore a promising tool to produce large number of true to type disease free plants in limited period of time and space (Sivaram and Mukundan, 2003; Tamura *et al.*, 1984). The demand of this plant has been increasing recently both in local and international markets. But reports on tissue culture of stevia

in Bangladesh is scanty. Hence, the study was undertaken for rapid multiplication and subsequent plantlet regeneration in stevia with the following objectives :

1. To identify the suitable sources of explants for micropropagation,
2. To find out suitable concentration of growth regulator for shoot proliferation and root induction,
3. To optimized suitable concentration of 2,4-D for callus induction and
4. To standardize the potting media for *ex vitro* establishment of stevia.

Plate 1. Stevia Plant



CHAPTER – II

REVIEW OF LITERATURE

CHAPTER II

REVIEW OF LITERATURE

The worldwide demand of stevia is rising day by day. A number of compounds present in the leaf tissue that have a potential use as low calorie sweetners to control blood sugar level. So, it received much attention of the researchers throughout the world to develop its propagation. Now a days, micropropagation is a rapid method which eliminates diseases and provide scope for large scale multiplication (Debergh and Read, 1990). There are many factors that govern *in vitro* organ development of stevia which includes type of explant, shoot quality, type and concentration of growth regulator etc. A large number of works have been done on sweetening status of Stevia by different form in different foods, but findings of those works have little relevance to micropropagation. However, some of the available research works related to present studies have been reviewed in this chapter.

USE AND STATUS OF STEVIA

Stevia is an obligate short day plant with a critical day length of about 13 h. Extensive variability within populations for day length sensitivity has been reported (Valio and Rocha, 1966).

Zaidan *et al.*, (1980) suggested that Stevia should be harvested just prior to flowering when steviol glycoside content in the leaves is at its maximum.

The leaves of *Stevia rebaudiana* are 30 times sweeter than sugar (Duke, 1993; Bhosle, 2004). It is also non-calorific, non-fermentable and does not darken upon cooking (Crammer and Ikan, 1986).

Eight diterpene glycosides with sweetening properties have been identified in leaf tissue of Stevia. The four major sweeteners are stevioside, rebaudioside-A, rebaudioside-C and dulcoside-A. The sweetness of those same compounds retative to sucrose is 210, 242, 30 and 30 respectively (Kinghorn, 1992).

Stevioside, the major sweetener present in leaf and stem tissue, was first seriously considered as a sugar substitute in the early 1970's by a Japanese consortium formed

for the purpose of commercializing stevioside and Stevia extracts (Kinghorn and Soejarto, 1985).

Total sweet glycoside concentration in some lines from China was reported to be as high as 20.5% and rebaudioside-A to stevioside ratio of 9:1 was disclosed in the Japanese patent literature (Shizhen, 1995; Morita, 1987).

The sweet compounds pass through the digestive process without chemically breaking down, making Stevia safe for those who need to control their blood sugar level (Strauss, 1995).

Recent clinical studies have shown it can increase glucose tolerance and decrease blood sugar levels. Of the two sweeteners (Aspartame and Stevia), Stevia wins hands down for safety (Whitaker, 1994).

Stevia has virtual no calories. It dissolves easily in water and mixes well with all other sweeteners. It was used in delicious homemade ice cream that was low in carbohydrates (Atkins, 1994).

The reproductive anatomy of the male and female gametophytes of Stevia is typical for angiosperms (Shaffert and Chetobar, 1994ab).

Literature related to safety of Stevia sweeteners concluded that Stevia leaves and stevioside are safe for human consumption (Kinghorn and Soejarto, 1985; Kinghorn, 1992).

In studies of acute toxicity, a LD_{50} of 8.2 g/kg for a refined stevioside extract and an acceptable daily Stevioside intake of 7.9 mg/kg were suggested by Xili *et al.*, (1992).

Drying Stevia under artificial conditions is affected by a number of factors including loading rate, temperature and ambient air conditions (Van Hooren and Lester, 1992).

Plants can initiate flowering after a minimum of four true leaves have been produced (Carneiro, 1990). Following harvest the whole plant is dried and the leaves separated from the stems for further processing (Murai, 1988).

Sweetening agents of Stevia are now in general use in Japan, China, Korea, Israel,

Brazil and Paraguay (Crammer and Ikan, 1986) and to date there have been no reports of adverse effects from the use of Stevia products by humans (Kinghorn and Soejarto, 1985).

Stevioside, the major sweetener present in leaf and stem tissue, was first seriously considered as a sugar substitute in the early 1970's by a Japanese consortium formed for the purpose of commercializing stevioside and Stevia extracts (Kinghorn and Soejarto, 1985).

Since Stevia is a self –incompatible (Miyagawa *et al.*, 1986) seed collected from an individual plant would represent a half- sib family (HSF).

Poor seed germination is one of the factors limiting large scale cultivation (Shock, 1982; Duke, 1993; Carneiro, 1990).

Suttajit *et al.*, (1993) found that stevioside and steviol are neither mutagenic nor clastogenic *in vitro* at the limited doses; however, *in vivo* genotoxic tests and long-term effects of stevioside and steviol are yet to be investigated.

TYPE OF EXPLANTS

Micropropagation has become a valuable and standard method for rapid clonal propagation (Murashige, 1974). Most economically acceptable methods for *in vitro* propagation are based on the capacity of initial explants to form clumps of multiple shoots which are subsequently divided manually several times to obtain enough shoots. These methods have been employed successfully for clonal propagation for a wide range of plant species including fruit, forest trees, medicinal plant and ornamentals (Murashige, 1978).

An advantage of shoot culture as compared to callus culture is that it takes a shorter time to induce shoots (Bonga, 1977). In culture, shoots retain their regenerative capacity over a long culture period.

The main disadvantage of shoot culture is that a single explant forms only a few shoots at a time, while hundreds or thousands shoots are sometimes produced in callus culture (Bonga, 1977). This disadvantage can be overcome by repeated subculture of

the explant. For example, Whitehead and Giles (1976) reported that one million *Populus* plantlets could be produced from a single axillary bud per year by repeated subculture. Abbott (1978) stated that the risk of producing mutant plants which is more common with callus culture is almost eliminated by using shoot culture. Thus, for clonal propagation shoot culture techniques should get priority over callus culture techniques (Murashige, 1974). This method is applicable to a wide range of plant species (Jones, 1983).

Shoot proliferation is common in angiosperms mostly from axillary buds. The use of buds is preferred to maintain genetic stability. Shoot cultures are initiated from explants which are either shoot tip or nodal explants. In higher plants shoot tip and nodal explants contain a bud or buds each of which is capable of growing into a shoot indentical to the mother plant (Hussey, 1983).

There were a number of reports on rapid shoot proliferation of stevia from shoot tip, leaf or nodal explants (Sivaram and Mukundan, 2003). Hoque and Fatema (1995) reported that maximum number of shoot regeneration was obtained using shoot tip explant in chrysanthemum. Kyte (1987) stated that African violet had surprising *in vitro* regeneration capacity from all parts of a leaf. Explants of petioles and leaf blades of *Saintpaulia ionantha* were cultured on various modifications of MS nutrient medium. Shoot induction was best in petiole explant.

Rapid multiplication of shoot was initiated from pedicel, thalamus, nodal segment and young shoot tip of gladiolus. The best performance was observed in MS medium with 2.0 mg/L BAP (Begum and Hadiuzzaman, 1995).

Plant regeneration from isolated pieces of leaf lamina was the most widely employed technique for *in vitro* propagation of African violet as reported in the literature by many researchers. Characteristically, callus begins growth at the cut surface of the explants and adventitious organs are produced from this material which may retain more or less green colour if light intensities and photoperiod are adequate (Start and Cumming, 1976).

Although shoot cultures can be established from shoot tips, most workers prefer nodal explants for multiple shoot formation. In stevia, Islam (2004) reported that shoot tip explants showed better proliferation than nodal explants. Acuna *et al.*, (1997) cultured

in vitro nodal segments of stevia on MS medium with 50% macroelement content and in the presence of 1.0 mg/L NAA and 2.0 mg/ l BAP induced the greatest shoot development with upto 10 shoots per explants.

The most effective explants for large scale production of the plants appeared to be micro-cuttings with apical or axial buds. Salas (2001) reported that apical buds of stevia were successfully cultivated on MS medium supplemented with 2mg/L BAP and 0.5 mg/L NAA and this allowed to producing the plants by several thousands in 3-4 months.

EFFECT OF GROWTH REGULATORS FOR SHOOT PROLIFERATION

A number of cytokinin compounds were used in axillary shoot proliferation. Those mostly used are Benzylaminopurine (BAP), Kinetin (Kn),Thidiazuron (TDZ) and 2-Isopentenyladenine (2ip). A specific cytokinin was sometimes better for a specific species of plant but BAP was much more active than naturally occurring cytokinin, such as 2ip, in proliferating shoots. While culturing shoot tip of stevia (Kabir, 2005) it was noticed that BAP was essential for the proliferation of axillary shoots and it was concluded that optimum concentration of BAP for axillary shoot proliferation was 3 mg/L . However, Goyal *et al.,* (1985) found that 2-3 mg/L BAP concentration induced the greatest shoot development with upto 5 shoots per explant in *Leucaena leucocephala.*

Chowdhary (1991) reported that BAP 2.5 mg/L was optimum for shoot multiplication of Indian rose. Begum and Hadiuzzaman (1995) stated that multiple shoot induction was best in half MS medium supplemented with 0.75 mg/L BAP. Grewal *et al.,* (1995) reported that nodal segments regenerated single shoot per explant upon culturing on MS medium supplemented with 1.0 mg/L BAP, whereas, those cultured on MS +5.0 mg/L BAP medium developed 14-20 shoots primordial within 4 weeks. TDZ at 1.0 mg/L induced high frequency shoot regeneration from immature cotyledons leading to the development of normal shoots (George and Eapen, 1999).

Kinetin also supports the growth of gladiolus explants (Ziv, 1979) and stimulates shoots proliferation. Again, Elliot (1970) found Kn ineffective of promoting the growth of rose tips. Similarly, Kabir (2005) reported Kn as less effective for stevia

shoot multiplication. Whereas, Fonnesbech *and* Fonnesbech., (1979) reported that 2ip and zeatin was better to promote the growth and survival of shoot tip culture of *Asparagus plumosus* than kinetin or BAP.

Some workers preferred to use a mixture of two or more cytokinins for shoot proliferation. In some rose cultivars like 'Crimson glory' and 'Glenfiditch', Brave *et al.*, (1984) reported that a combination of BAP and Kn at a concentration of 0.5 and 0.2 mg/L, respectively developed healthy green multiple shoots (2-6 shoots/explant) within 15-20 days.

Sivaram and Mukundan (2003) regenerated shoots from shoot apex, nodal, and leaf explants of stevia by culturing them on Murashige and Skoog (MS) medium supplemented with 6-benzyladenine (BA) 8.87 μM and indole-3-acetic acid (IAA) 5.71 μM.

Kabir (2005) developed the procedures for micropropagation from primary leaf and shoot tip of stevia. Shoot multiplication from shoot tips were observed on MS medium supplemented with 2.0 mg/L kinetin.

A comparative study of the effect of BAP and thidiazuron (TDZ) on shoot bud differentiation from excised cotyledons of Brassica juncea cv. PR-45 showed that the later is ten times more effective than the former and in combination produced synergistic effect. Nidhi (1998) at its optimum concentration (5×10^4 mM) of TDZ induced 100% regeneration with 7.4 shoots per cotyledon.

Rida *et al.*, (1999) produced an efficient procedure for production of disease free carnation (*Dianthus caryophylus*) cv. 'Balady'. Highest shoot formation was obtained on medium supplemented with 1.0 mg/L 6-benzylaminopurine (BA) and 0.1 mg/L 1-naphthalenacetic acid.

Venkatachalam *et al.*, (1998) has been achieved shoot bud multiplication in *Eucalyptus camaldulensis* using nodal explants from 15-years-old elite trees growing in field. Of the two cytokinins tested, BAP was very effective for induction of shoots and the best concentration was 1.5 mg/L. Results showed that addition of NAA at 0.5 mg/L induced maximum number of shoots buds.

Different explants, namely cotyledonary node, stem node and shoot apex of *Leucaena*

leucocephala were cultured on MS medium supplemented with 2.5 mg/L BAP and 50-200 mg/L casein hydrolysate. All explants produced multiple shoots. Maximum number of multiple shoot production was obtained from cotyledonary node explants on MS medium supplemented with 2.5 mg/L BAP and 150 mg/L casein hydrolysate (Ara *et al.*, 1991)

Multiple shoot regeneration was tried by Hoque *et al.*, (1996) from nodal and shoot tip explants of carnation (*Dianthus caryophylus* L.) on MS and modified half MS medium containing different concentrations of BAP, NAA and Kn. Of the different combinations of BAP and Kn used, best response was achieved in half MS medium containing 1.5 mg/L BAP and 0.5 mg/L Kn. In this combination 10-12 shoot buds were initiated from a single explant and the number of multiple shoots increased at least two-threefold within 10-14 days when they were subcultured in the same medium. On the other hand, shoot tip explant produced 8-9 shoots per explant in MS medium containing 0.75 mg/L BAP and 0.2 mg/L NAA.

Ara *et al.*, (1991b) reported that cotyledonary nodes of *Leucaena leucocephala* showed best response in MS medium containing 1.0 mg/L BAP than other explants such as shoot tip, hypocotyl and cotyledon segments used for *in vitro* multiple shoot regeneration.

EFFECT OF GROWTH REGULATORS FOR CALLUS INDUCTION

The development of protocols for regeneration of *Stevia rebaudiana* via somatic embryogenesis is important as this technique can be used in the clonal propagation of this plant, or as explant material for protoplast isolation and regeneration (Puite, 1992). Sometimes the addition of auxin helps promoting the development of axillary shoots. He also studied callus and shoot proliferation of stevia by using various combinations of growth regulators.

The optimal conditions for callus induction from primary leaf of stevia were observed on MS medium supplemented with 2.0 mg/L NAA and 2.0 mg/L BA by Duke (1993). The optimal conditions for primary leaf callus regeneration was achieved on MS medium containing Nitsch and Nitsch (1969) vitamins supplemented with 2.0 mg/L NAA and 2.0 mg/L BA. The efficiency of adventitious shoot formation could be

increased a number of 60 shoots per callus within 3 months.

Mature leaf segments of *Saintpaulia ionantha* cv. Raving Red on MS culture medium supplemented with 2.0 mg/L 2, 4- D formed callus which latter differentiated into green shoots (Cooke, 1977).

The best callus formation and shoot differentiation in the cultivars "Blue Boy" and California" of African violet were obtained on modified MS medium containing 1.0 mg/L BA and 0.25 mg/L NAA in a 4:1 ratio BA or NAA alone or together stimulated the formation of roots and shoots. The best plantlet growth was obtained with 2.0 mg/L BA+0.05 mg/L 2, 4-D (So, 1983).

Tissue culture of the winged bean were studied by Evans *et al.*, (1981) to determine their callus induction ability and plant regeneration potential using leaf as explants in MS medium. The best callus induction was achieved on MS medium with 0.2 mg/L NAA and 2.0 mg/L BAP. Development of adventitious shoots occurred when the calli were subcultured in MS medium supplemented with IAA and Kn. Maximum frequency of calli (60%) induced adventitious shoots and highest number of shoots per callus were obtained when the medium was fortified with 2.0 mg/L Kn and 2.0 mg/L IAA.

Islam *et al.*, (1992) reported that regeneration of multiple shoots via callus induction and organogenesis was achieved from leaves of *in vitro* grown shoots of *Aegle marmelos*. The presence of 2, 4-dichlorophenoxyacetic acid (2, 4-D) in the culture medium at 1-5 mg/L resulted in callus growth.

Islam (1999) reported that calli of the three cultivars eg, *Phalaenopsis, Doritaenotsis* and *Newfinetia* grown on maltose and sorbitol medium, initiated protocorm like bodies (PLBs) which subsequently developed into plantlets. Regenerated plantlets were normal and healthy in appearance.

Chang and Chang (1997) stated that totipotent calli of *Cymbidium ensifolium* var. *misericors* (a locally grown orchid of high commercial value) were induced from sections of pseudo bulbs, rhizomes and roots of seed derived plantlets on half strength

Murashige and Skoog medium plus 10.0 mg/L 2, 4-D and 0.1 mg/L thidiazuron. The calli were maintained by subculturing in the same medium.

Chen *et al.*, (2000) established an efficient method for high frequency somatic embryogenesis and plant regeneration from callus cultures of a hybrid of sympodial orchid (*Oncidium* Gower Ramsey). Initiation of somatic embryogenesis and development up to the protocorm like bodies (PLBs) from callus cultures was achieved on hormone free basal medium. Regenerants were recovered from somatic embryos (SEs) after transfer to the same medium and showed normal development. The plant produced more attractive colour and longer shelf than Oncidium Gower Ramsey.

ROOT DEVELOPMENT *IN VITRO*

It is a general observation in micropropagation that the induced shoot fails to develop roots at the shoot proliferation phase. A separate root induction phase is, therefore, essential.

Effect of growth regulators for rooting

Most plant species require the supplement of one or more growth regulators in the medium for root initiation. The most commonly used auxins are Napthaleneacetic acid (NAA), Indole-3-butyric acid (IBA) and Indole-3-acetic acid (IAA). Sometimes one auxin appeared much better that of another for a specific species of plant. In stevia, Kabir (2005) found that the addition of NAA in the medium produced roots. IAA was less effective while IBA was intermediate in effect. Similarly, NAA was better than IBA in *Carambola* L. and *Ficus religiosa* at 0.5 mg/L (Hakim *et al.*, 1995). Whereas in rose, the preferred auxin for root induction was IAA (Bressen *et al.*, 1982; and Sauer *et al.*, 1985). Nevertheless, when IAA was used in combination with NAA better rooting response was obtained by Ellyard (1981). It is now well known that rooting of plants may proceed best when the overall salt strength of the medium is reduced. In some cases the salt strength reduction may eliminate the need for auxins for rooting. Skirvin and Chu (1979) reported that good rooting of 'Forever Yours' rose could be obtained on quarter strength Murashige and Skoog (1962)

mineral salts supplemented with only full strength sucrose (30 g/l). Davies (1980) reported that he could obtain 100% rooting of several cultivars of rose by placing them on MS medium supplemented with 40 g/l sucrose without growth regulator. Khosh-Khui and Sink (1982) reported that half-strength MS medium supplemented with NAA (0.54 µM) will do the adequate job of rooting in 'Bridal Veil Hybrid' rose. Similar results have been reported by Hasegawa (1979) who found that the improved 'Blaze' rose rooted well on quarter to half strength MS medium supplemented with IAA (1.7 µM) or NAA (1.6 µM).

Fonnesbech and Fonnesbech (1979) reported that the optimal condition for root induction in asparagus was occurred both in MS medium supplemented with 0.01 mg/L NAA and MS medium without plant growth regulator. Plantlets were successfully transplanted to potting soil.

Morel and Muller (1964) reported that mineral salt medium containing NAA and IBA at the rate of 0.05-1.0 mg/L with 16 g/l sucrose is essential for adventitious root formation on petioles and lamina of African violet. In chrysanthemum, the preferred auxin for root induction was IAA (Jain and Chaturvedi, 1993; Hoque and Fatema, 1995).

Acuna *et al.*, (1997) reported that mineral salt medium containing NAA and IBA at the rate of 0.05-1.0 mg/L with 30 g/l sucrose is essential for adventitious root formation on stevia. At the transfer of plants from *in vitro* into *in vivo* conditions nodal segments were dipped in a 5% IAA solution to promote rooting. Treated plants were grown for 1 month in a greenhouse and then planted into the field. A most effective preparation for increasing the concentration of stevioside in leaves was Humiforte (synthetic amino acids, N, P, K and trace elements) in combination with Aminol (amino acids and N) but Melatran (lactic and anthranilic acids) gave the highest biomass yields.

Kashem (1992) reported that 100% rooting was observed of several gladiolus cultivars within 15-20 days when 1.0 mg/L IBA were used in 1/2 MS medium. Dantu and Bhojwari (1987) used BM and MB+BAP (0.1, 0.2 mg/L) and 33% shoots were rooted in within a week. Wilfret (1971) studied that root production was higher in

Gladiolus in the Murashige and Skoog formulation as compared to the Linsmaier and Skoog media. Kamo *et al.*, (1990) found that the regenerated plantlets of Gladiolus formed roots when transferred to a MS basal salt medium lacking hormones. Begum and Hadiuzzaman (1995) reported that in Gladiolus, healthy roots were produced in half MS medium supplemented with 0.5 mg/L IBA. Fridborg and Eriksson (1975), reported that AC in the rooting medium has a beneficial effect on shoot growth as well as root length.

Rida *et al.*, (1999) produced an efficient procedure for production of disease free carnation (*Dianthus caryophylus*) cv. 'Balady'. Maximum rooting was achieved on medium containing 1.0 mg/L indole-3-butyric acid (IBA) with 0.1 g/L activated charcoal (AC) or 0.1 g/L phloroglucinol (PG). Rooted plantlets were transferred to greenhouse in 1 peat: 1 perlite mixture.

Tissue culture of the winged bean were studied by Evans *et al.*, (1981) to determine their callus induction ability and plant regeneration potential using leaf as explants in MS medium. Rooted plantlet formed 10-14 days after transfer from MS medium containing 0.2 mg/L IAA and 2.0 mg/L BAP to MS medium with reduced sucrose (0.5%) and 0.4 mg/L NAA. Mature plants were transferred to the field and produced flowers and fruits.

Venkatachalam *et al.*, (1998) has been achieved rooting on regenerated shoots of Eucalyptus. Rooting was best on half-strength MS medium containing 1mg/L IBA. The regenerated plants have been successfully acclimatized and transferred to soil. About 45% of the plants have survived under *ex vitro* condition. This system could be beneficial for mass propagation of selected elite clones.

Islam *et al.*, (1992) reported that carambola shoot developed roots when they were put on half strength MS medium with 1.0 mg/L of indole-3-butyric acid (IBA).

Best response towards healthy rooting of carnation (*Dianthus caryophyluus* L.) was achieved on 0.2 mg/L IBA and 0.2 mg/L NAA used in MS medium solidified with phytagel. The plantlets from the test tubes were transferred and established in the soil.

Ara *et al.*, (1991b) reported that in *vitro* individual shoots were separated out and cultured in rooting medium i.e. half the strength of MS medium supplemented with 0.2 mg/L IBA. The well developed plantlets of *Leucaena leucocephala* were transferred and established in the soil.

EX-VITRO ESTABLISHMENT

Successful establishment of *in vitro* cultured plantlets in *ex vitro* conditions plays a significant role in the success of tissue culture propagation techniques. Establishment of cultured plantlets in the greenhouse or in the field depends upon the development of suitable conditions to harden the plantlets rapidly. *In vitro* cultured plantlets show several abnormalities which affect the survival of plantlets in the *ex vitro* environment conditions and nature of soil mixes.

The nature or type of substrate used for transplanting *in vitro* plantlets to *ex vitro* environment varies from species. Stone (1963) reported that carnation plantlets grew better in a mixture of peat: loam (1:1:1: v/v) covered with a layer of peat and sand than perlite or vermiculite mixes. *In Rhododendron* spp. (Anderson, 1978) fertilizer inclusion to the soil mix greatly improved survival, growth and development of plantlets. Skirvin and Chu (1979) transplanted 'Forever Yours' hybrid rose plantlet to clay pots with vermiculite with glasscover to prevent desiccation. Uddin (1993) observed that 85% success in vermiculite and an estimated 71% success in soil with tissue cultured plantlets of thuja.

Plantlets of African violet were established easily in a soil mixture of 2 peat: 2 perlite: 2 fir bark: Sand in 5 cm pots without mist spray (Cooke, 1977).

Rao (1985) reported that terrestrial orchids like *Spathoglottis, Paphiopedilum, Phaius* and *Calanthe* had grown well in 20-25 cm pots with 1: 1: 1 mixture of leaf mould, FYM and sand.

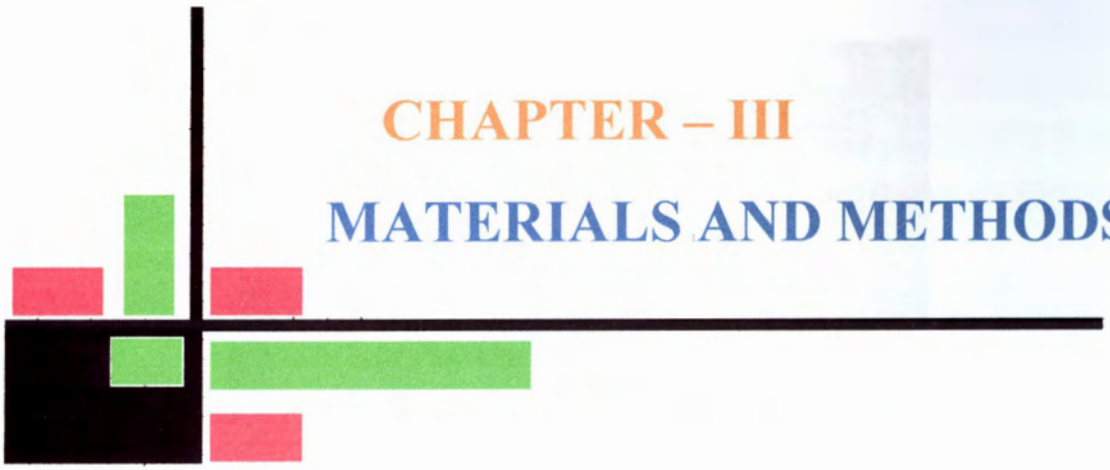
It is revealed from a botanical survey of India that in case of *Rhyncostylis gigantea*, chunks of hard-wood charcoal alone as potting substrate were superior to other potting media (Bhattacharjee, 1985). Tree fern fibres performed better than the other media. But coconut husk and over burnt brick as planting substrates had adverse effects on growth and flowering of *R. gigantea* plants.

Raju *et al.,* (1997) hardened the *in vitro* rooted *Dendrodium* plantlets using coir dust, perlite and vermicompost in the ratio of 1:1:1 (v/v) under mist conditions and successfully established in the Orchidarium.

Biswas *et al.,* (1994) evaluated the effect of different potting media on growth and flowering behaviour of two orchid species, each of epiphytic (*Aerides multiflorum, Dendrobium moschatum)* and terrestrial *(Cymbidium aloifolium, Phaius tankervillae)* They mentioned that the pot medium comprising charcoal brick pieces, coconut husk and tree fern was suitable for the epiphytic orchids. Further, a mixture of loamy soil, leaf mould, river sand, tree fern, charcoal dust and old mortar was the best for growth and flowering of terrestrial orchids.

According to Bhattacharjee and Mukherjee (1981), monopodial epiphytic orchids performed best when grown in chunks of hardwood charcoal only, while sympodial epiphytes did better in osmunda tree fern fibre. Terrestrial orchids performed well in organic rich porous compost mixture.

Alam (2003) reported that the plantlet height in *Phaius* was greatly influenced by potting media. The longest plant was recorded in the potting medium of 33% compost + 33% sand + 33% soil.



CHAPTER – III

MATERIALS AND METHODS

CHAPTER III

MATERIALS AND METHODS

Two experiments were conducted at Biotechnology Laboratory, Bangladesh Sugarcane Research Institute (BSRI), Ishurdi, Pabna, Bangladesh during the period of January – October/2006.

Plant materials

Shoot tip, leaf base and nodal segments (Plate 2) were used as explants for shoot regeneration of direct *in vitro* propagation. Young leaf disk, mature leaf disk and stem node were used for callus induction. All the explants were collected from 6-8 months old yard grown plants at Biotechnology Laboratory, BSRI.

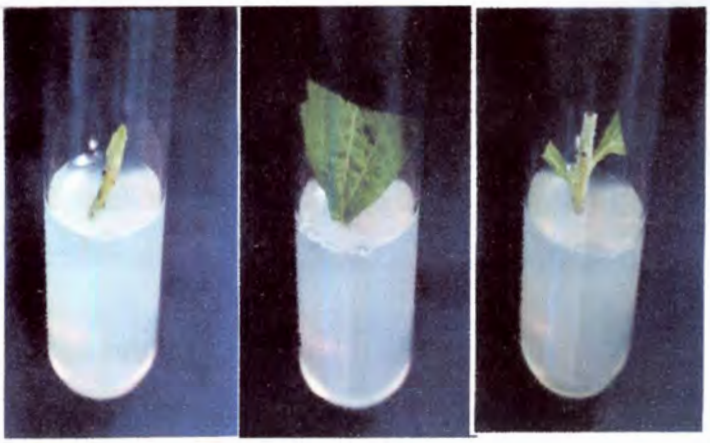


Plate 2. Explants (from left- shoot tip, leaf base with petiole and nodal segment) were inoculated on media in test tube for shoot regeneration

Culture media

Murashige and Skoog (1962) medium was prepared following the composition mentioned in Table-I and media preparation was carried out following the different steps mentioned below.

Stock solution preparation

Prior to medium preparation, stock solutions of different components were prepared. Five stock solutions like stock solution of macronutrients (MS I), micronutrients (MS II), iron source (MS III), Vitamins (MS IV) and plant growth regulators were prepared.

Stock solution of macronutrients

Stock solution of Macronutrients was prepared 10 times of desired concentration with distilled water. Required amount of salts were taken in a conical flask, following the serial number mentioned in Table-1 and were dissolved in distilled water using a magnetic stirrer. Finally, required distilled water was added up to the mark. Prepared solution was kept in a glass container, labeled with marker and stored in a refrigerator at 8 - 9^0C for use.

Stock solution of micronutrients

The stock solution of micro nutrients was made up to 100 times higher strength than required concentration of the medium in one liter of distilled water as described earlier for the stock solution of macro nutrients. This stock solution was also filtered and stored in refrigerator at 8 -9^0C.

Stock solution of iron source

The stock solution of iron source was made 100 times the final strength of the medium in one liter of distilled water. Here, two constituents, $FeSO_4$ and Na_2 EDTA, were dissolved in 750 ml of distilled water in a conical flask by heating in a water bath until the salts dissolved completely and final volume was made up to one liter by further addition of distilled water. This stock solution was also filtered and stored in refrigerator at 8 - 9^0C.

Stock solution of vitamins and amino acids

The vitamins and amino acids such as pyridoxine HCl (Vitamin B_6), Thiamin HCl (Vitamin B_1), Glycine and Nicotinic acid (Vitamin B_3) were used in the preparation of MS medium. The stock solution of vitamins and amino acid were prepared 100 times the concentration of their final strength and stored at 8-9^0C.

Stock solution of plant growth regulators (PGR)

Stock solution of PGRs were prepared by dissolving the desired quantity of ingredients to the appropriate solvent and made the final volume with distilled water. The following growth regulators were used in the present investigation.

a) **Cytokinin**

6-Benzylaminopurine (BAP)

6-Furfurylaminopurine (Kn)

2-Iso pentenyladenine (2ip)

b) **Auxins**

Indole-3-butyric acid (IBA)

α-Napthaleneacetic acid (NAA)

Indole-3-acetic acid (IAA)

2, 4-Dichlorophenoxy Acetic Acid (2, 4-D)

The growth regulators were dissolved in appropriate solvents as follows:

Growth regulator (solutes)	Solvents
BA	0.1N HCl
Kn	0.1N HCl
2ip	0.1N KOH
IBA	0.1N KOH
NAA	0.1 KOH
IAA	0.1N KOH
2,4-D	0.1N KOH

To prepare the stock solution, 10 mg of growth regulator was taken in a 250-ml clean beaker and then dissolved in 1 ml of respective solvent mentioned above. Then the volume was made up to 100 ml by adding distilled water to get 100 mg/L solution. The prepared hormone stock solution was then stored at 8-9^{0}C for subsequent use.

Steps followed for the preparation of MS culture media (1 Liter)

i) The required volume of each stock solution (100 ml macro nutrients, 10 ml iron source, 10 ml micro nutrients, 10 ml vitamin) were poured into a beaker and mixed.

ii) About 200 ml distilled water was added to this.

iii) 30 mg of sucrose was added to this solution and gently agitated with the help of a magnetic stirrer to dissolve completely.

iv) This solution was poured into beakers taking 250 ml of the solution in each beaker. Required volume of PGR solution for each treatment was directly added to the solutions in the beakers.

v) Adding distilled water treatment wise and final volume was made.

vi) The pH of the solutions was adjusted to 5.7 with a digital pH meter adding either 0.1 N NaOH or 0.1 N HCl as and when necessary.

vii) After adjusting the pH, agar was added in each beaker. Each mixture was then heated in a microwave oven till complete dissolution of agar took place. Care was taken so that the solution did not get boiled while melting agar.

viii) About 10 ml and 20 ml of warm media were dispensed into test tube and vials and were closed with a piece of polythene sheet and rubber band and marked to indicate specific treatment.

To prepare 1litre of MS half strength same procedure was followed but the amount of stock solution and sucrose was reduced to half

Sterilization

Sterilization is a prerequisite for *in vitro* techniques. So all instruments, glassware, explants and culture media were sterilized.

Sterilization of glassware and other instruments

Beakers, conical flasks, pipettes, petridishes, metal instruments such as forceps, scalpels, needles, spatulas were sterilized by wrapping with aluminum foil in an autoclave at a temperature of 121^0C for 20 minutes at 1.06 kg/cm^2 pressure.

Sterilization of culture media

The culture vessels containing the media supplemented with 3 % sucrose and solidified with 0.7 % agar. The pH of the media was adjusted to 5.8 before autoclaving. The media were autoclaved with 1.06 kg/cm^2 of pressure at 121^0C for 20 minutes. After autoclaving the culture vessels containing the culture media were allowed to cool.

Sterilization of culture room and transfer area

The culture room was initially cleaned by gently washing all the floors and walls with a detergent and germicide like Savlon or Detol. This was followed by carefully wiping them with 70% ethyl alcohol.

The process of sterilization was repeated at regular intervals. Generally laminar airflow cabinet was sterilized by switching on the cabinet with UV light and wiping the working surface with 70% ethyl alcohol.

Culture Techniques

The culture techniques employed were-

a) Explant culture
b) Subculture
c) Incubation
d) Shoot induction
e) Root induction

Explant culture

Different plant parts like shoot tip, leaf base, leaf disc, internodal segments and nodal segments were used as explants for shoot regeneration of direct and indirect *in vitro* propagation. The explants were placed in autoclavable jar with water, few drops of tween-20 , cut into small pieces (about 1 cm long) and finally treated by $HgCl_2$ (0.1%)

for surface sterilization. Then, they were washed inside the clean bench 4-5 times with sterilize distilled water to remove chloride. Finally they were inoculated aseptically for *in vitro* propagation on MS (Murashige and Skoog, 1962) medium with different concentrations of growth regulators.

Subculture

Successful shoot formation became evident when small green leaves began to emerge. It is the first sign of regeneration. These tiny leaves when developed in their actual shape they were transferred into fresh media containing the same hormonal combination or best one among them for further proliferation and development. Subculture was carried out regularly at an interval of 4-5 weeks.

Incubation

For growth and development of cultures, the temperature was set 25 ± 1^0C at a light intensity of 3000 lux and the photoperiod maintained 16 hours light and 8 hours dark.

Shoot induction

The regenerated shoots were very carefully rescued from the culture tubes placed on a hard paper, cut from each basal end of the shoots, then each of the shoot was cultured to different combinations of hormonal supplements for shoot induction.

Root induction

The proliferated multiple shoots were separated and individual shoots were placed in ½ MS media containing various concentrations and combinations of IBA, NAA and IAA.

Acclimatization

After eight weeks of rooting, the rooted plantlets were brought out of the controlled environment of growth chamber for 5-7 days to bring them in contact of normal temperature. The plantlets were covered with a polythene bag to prevent sudden desiccation and kept in a shaded area (Plate 3) and watered every 48 hours. The plants were slowly introduced into direct sunlight and after 10-15 days the plastic bags were removed.

Plate 3: Hardening of Plantlets

Plantation of Plantlets

After 2-3 weeks of hardening of plant-lets they became 7-12 inches in height and strong enough for plantation under normal condition. Therefore, they are then transplanted to pots with different substrates. Care was taken of so that roots did not get any damage during plantation.

Experiments conducted during the study are outlined as follows:

Expt.1. Effect of type of explants and various levels of BAP on the proliferation and elongation of shoots

This experiment was designed with 3 types of explants- shoot tip, leaf base, and nodal segments (each of 1 cm size), and BAP at 0, 1.0, 2.0, 3.0, 4.0 and 5.0 mg/L. Three replication were used in each treatment and 5 tubes per replication were maintained.

Expt.2. Effect of cytokinins on the proliferation and elongation of shoots

Since in the previous experiment nodal explants gave better proliferation of shoots than shoot tips and leaf base; nodal explants were used in all further experiments. The experiment was designed with BAP, 2ip and Kn at 0, 1.0, 2.0, 3.0, 4.0 and 5.0 mg/L concentrations. There were 3 replication in each treatment and 5 tubes per replication. Proliferation of shoots was evaluated by counting number and measuring the length of shoots produced from each culture after 6 weeks.

Expt. 3. Effect of auxins on root formation and elongation

The influences of auxins for rooting efficiencies on explants applying various auxins namely indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) and α naphthalene acetic acid (NAA) at 5 concentrations (0.5,1.0, 1.5, 2.0 and 5 mg/L). The ½ MS media was solidified with 8 g/l agar and adjusted to pH 5.8. There were 3 replications in each treatment and 5 tubes per replication. The shootlets of 3 ± 0.5 cm were used as explant. Data were recorded after 8 weeks of culture.

Expt. 4: Effects of 2, 4-D and different explants on callus initiation.

There were two factors: a) Concentrations of 2, 4-D (level 7): 0.0, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0 mg/L and b) Type of explants (level 3): Mature leaf disk, younger leaf disk and stem node. Thus there were 21 treatment combinations in the experiment. There were 4 replication in each treatment and six tubes per replication. Percentage of callus initiation were recorded at 20, 30 and 40 days after inoculation. Morphogenic response of callus was recorded after 7 weeks.

Expt 5: Effect of NAA and BAP on growth and development of regenerated plantlets

Callus (0.3 g) cultured on MS basal medium and the medium was supplemented with different concentration and combination of NAA and BAP to observe their effect on the subsequent growth and development of plants. Three replications were used for each treatment and each vial containing six samples. Data were recorded 30 days, 60 days and 90 days after inoculation (DAI).

Expt.6. Effect of different substrates for *ex vitro* establishment of stevia

The healthy rooted plantlets were removed from culture medium and were transferred to pots containing different substrates viz. S_1 (100% soil), S_2 (50% soil + 50% cowdung), S_3 (100% cocodust), S_4 (50% cocodust + 25% soil + 25% cowdung), S_5 (25% cowdung + 25% cocodust +25% soil +25% saw dust). There were 3 replicates in each substrate and 10 pots per replication were used. Data on leaf number shoot number, plant spread, plant height, and growth response and survival percentage on substrates were recorded after three months.

Data collection

Data were collected on following parameters and the methods for data collection are given below:

i) Number of shoots per explant

Number of shoot per explant were counted after 6 weeks of culture. Arithmatic mean was calculated using the following formula.

$$\overline{X} = \frac{\Sigma Xi}{n}$$

$\overline{X}$ = Mean number of shoot/explant

Σ = Summation

Xi = Number of shoot/explant

n = Number of observations

ii) Length of shoot

Length of shoot was measured in centimeter for each explant. Average length of the shoot was found out using the formula like previous calculation.

iii) Number of roots per shoot

Shoots were cultured separately in 120 ml culture tubes for each of the combination of media with rooting hormones. Shoots which were induced to develop roots were taken out after 10 weeks of culture and average number of roots per shoot was calculated.

v) Callus induction, Growth and development of plant lets

Embryogenic callus was recorded after 7 weeks. Data were recorded on the following parameters.

a) Success percentage of callus initiation
b) Morphogenic response of callus
c) Number of shoots
d) Number of roots etc.

(v) Establishment of plantlets to different substrate

The percentages of plantlets established in different substrate were calculated using following formula.

$$\% \text{ of success of plantlets} = \frac{\text{Number of plantlets established in substrate}}{\text{Total number of plantlets placed in substrate}} \times 100$$

(vi) Growth response of *ex vitro* plant lets

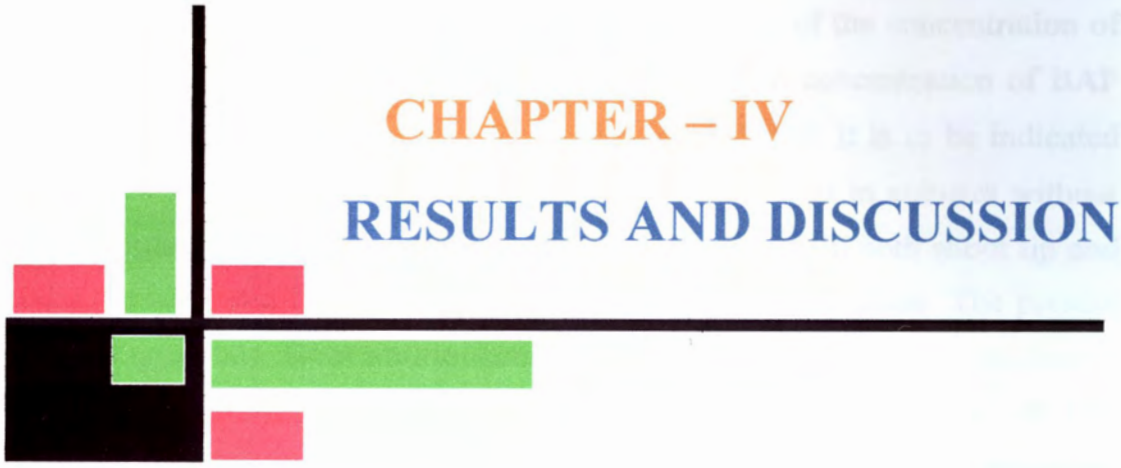
The scoring of growth response was determined based on eye estimation poor, good and excellent in terms of vigour and colour of leave and shoot respectively.

Design

The experiments were set up in Completely Randomized Design.

Statistical analysis

The collected data for various characters were statistically analyzed using MSTAT-C Computer package programme. The mean for all the treatments were calculated and the analysis of variance for each of the characters were performed by F-test.



CHAPTER – IV

RESULTS AND DISCUSSION

CHAPTER IV

RESULTS AND DISCUSSION

Experiment 1. Effect of type of explants and BAP on the proliferation and elongation of shoots

The explants, shoot tip, leaf base with petiole and nodal segment of stevia were cultured on MS Medium supplemented with 5 different concentrations (0.0,1.0, 2.0, 3.0, 4.0 and 5.0 mg/L) of BAP. The results presented in table 1 showed that shoot proliferation was influenced by the type of explants. Among the explants shoot tip and nodal segment were able to produce multiple shoots. Nodal segment initiated shoots earlier than shoot tip explants. Leaf base with petiole failed to produce shoot on any of the concentration of BAP used. There was significant interaction between concentration of BAP and type of explants on the number of shoots produced. It is to be indicated here that a less number of multiple shoots were obtained in cultures without BAP and the highest concentrations of BAP (5.0 mg/L) in both shoot tip and nodal explants and the regenerated shoots were weak in nature. The present findings regarding shoot multiplication at control and higher concentration of BAP showed similarity with the reports Hasegawa (1979). Out of five different concentration of BAP tried, the shoot proliferation and elongation was increased in both explants with 3.0 mg/L (Table2). However, the highest number of shoots and elongation was produced by node (6.8 and 6.4 cm) with 3.0 mg/L BAP followed by 2.0 mg/L BAP (5.3 and 5.0 cm) respectively. Islam (2004) reported that 3.0 mg/L BAP was optimum for shoot proliferation and elongation of stevia used for both the explants. Therefore the results of the present investigation were supported with the findings of the above worker.

Table 1. Influence of types of explant and BAP on shoot proliferation and elongation of stevia

Explants	MS+BAP (mg/L)	Days of Shoot initiation	Shoot number per culture	Shoot length (cm)
Shoot tip	Control	26±0.3	2.0±0.5	2.0±0.4
	1.0	24±0.9	3.2±0.7	2.9±1.0
	2.0	25±0.8	4.0±0.9	3.7±0.6
	3.0	20±1.0	5.3±1.3	5.0±0.8
	4.0	22±0.6	3.4±0.5	2.8±0.5
	5.0	24±0.5	2.1±0.6	2.0±0.3
Node	Control	23±0.4	2.2±0.8	2.0±0.6
	1.0	21±1.0	3.5±1.0	3.0±0.9
	2.0	20±0.6	5.1±1.2	4.5±0.7
	3.0	17±0.9	6.8±0.9	6.4±1.2
	4.0	20±0.5	4.4±1.1	3.2±0.9
	5.0	22±1.0	2.0±0.4	2.1±0.5
Leaf base	Control	-	-	-
	1.0	-	-	-
	2.0	-	-	-
	3.0	-	-	-
	4.0	-	-	-
	5.0	-	-	-

There were 3 replicates in each treatment and 5 tubes per replication. Data ($\overline{X} \pm SE$) were taken after 6 weeks of culture.

In explants, the endogenous hormonal concentrations in the node is lower than shoot tip. Addition of BAP during shoot proliferation further increases the hormonal level. The decrease in number of shoot in shoot tip might be due to adverse effect of excess hormone. Murashige (1974) reported that this variation might be attributed to the physiological difference of the various explant and differentiated expression of various tissues.

Plate 4. Development of proliferated shoots from nodal explant after 6 weeks of culture

Experiment 2. Effect of cytokinins on the proliferation and elongation of shoots in stevia

Cytokinin was essential for multiple shoot formation and elongation. Various concentrations of BAP, 2ip and Kn ranging from 1.0 to 5.0 mg/L along with control were used singly in MS basal medium to observe their effect on multiple shoot regeneration and elongation from nodal explant. Results of the study have been presented in Figures 1 and 2. It was observed from both Figure 1 and 2 that the type of cytokinin and their concentration had a significant effect on shoot proliferation and elongation in stevia.

Among the five concentrations, the shoot proliferation and elongation increased irrespect of cytokinin when applied 3.0 mg/L (Plate 4). However, the highest response was noted when BAP alone was used which produced 8.0 shoots within 15 weeks of culture (Fig. 1). The highest shoot length (8.7 cm) was also found with the application of BAP 3.0 mg/L. More or less similar result was observed in stevia by Salas (2001) and Kabir (2005). The second highest response was recorded in 2.0 mg/L BAP.

Multiple shoots were also obtained in control treatment and the highest concentration (5.0 mg/L) of all cytokinin (Fig. 2) but in both concentrations, elongation of shoots was poor, weak in nature and ultimately they turned green to brown in colour.

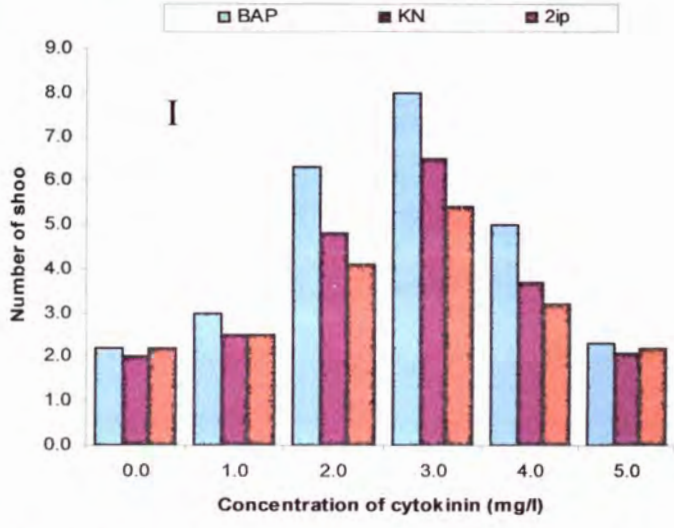


Fig. 1. Influence of cytokinins on the number of stevia shoot proliferation after 6 weeks of culture. Vertical bar represent LSD value at 1% level of probability.

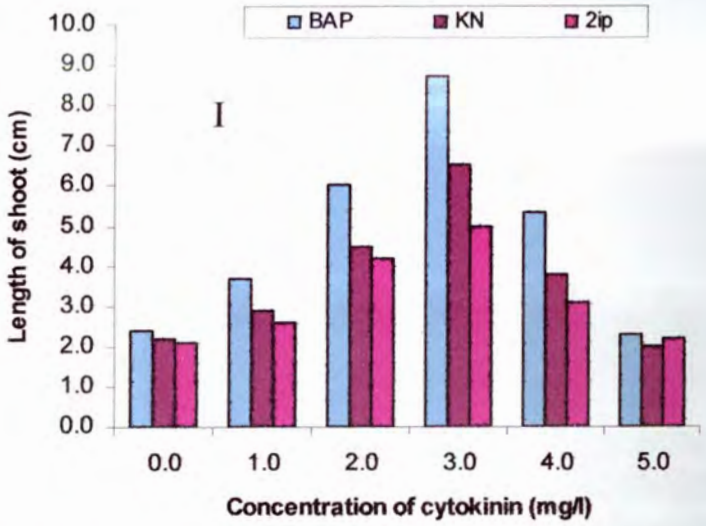


Fig. 2. Influence of cytokinins on the length of stevia shoot after 6 weeks of culture. Vertical bar represent LSD value at 1% level of probability.

The experiment with various cytokinins showed that BAP was more effective than Kn and 2ip. Ara *et al.*, (1992) showed that shoot proliferation of guava with BAP was superior to that with either Kn or 2ip which is in consonance with the present findings. The reason for increased proliferation with BAP might be due to the difference in activity of different cytokinin such as BAP is much more active than naturally occurring cytokinins, such as 2ip, in proliferating shoots which could be explained on the basis of degradation of 2ip by enzymes, whereas BAP was not (Ara, 1997).

The study showed that BAP concentration of 3.0 mg/L performed better than higher concentration (5.0 mg/L) as well as control for shoot proliferation and elongation of stevia. Inhibition of elongation with control and higher BAP concentrations might be due to the supra-optimal level, a response similar to that observed in rose (Ara, 1997).

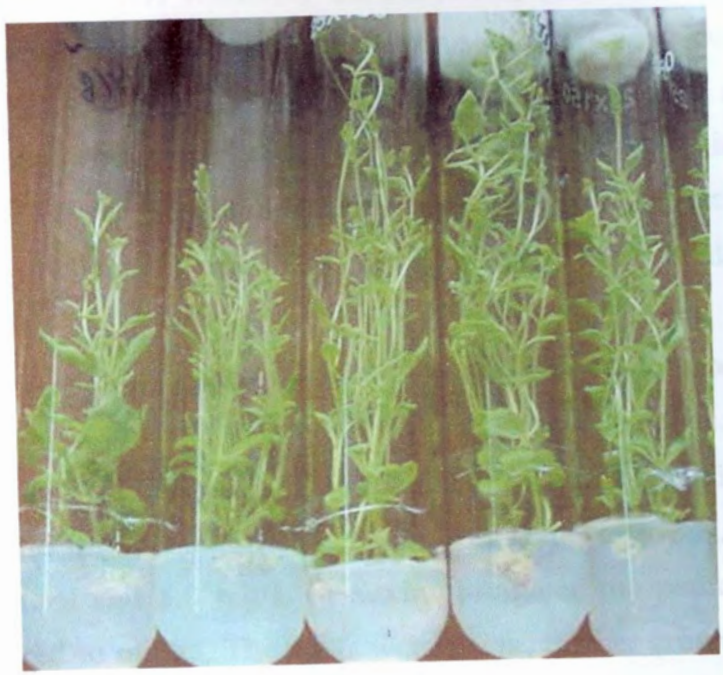


Plate 5. Proliferation and elongation of stevia shoot at 3.0 mg/L BAP after 6 weeks of culture

Experiment 3. Effect of different auxins on root formation of stevia

An auxin is often essential for *in vitro* rooting of many plant species. Excised shoots collected from in vitro grown materials and were cultured into ½ MS media having 5 concentrations of IBA, IAA and NAA ranging from 0.5 to 5.0 mg/L. The experiment included a control which received no auxin.

Percentage of root induction was markedly influenced by the concentration and nature of auxin. Among the three types of auxin used, NAA was found to be the best for percentage of root induction (Table 2). The next suitable auxin was IBA. 100% shoots produced shoot when they were cultured in the medium with 1.5 mg/L NAA. Media containing IAA also favored initiation and development of roots which were not as good as in NAA and IBA supplemented media.

The data present in table 2 also showed that NAA at 1.5 mg/L produced significantly higher number of roots than all other auxin concentrations. Each shoot produced 7.5 roots within 8 weeks of incubation in treatment with 1.5 mg/L NAA. The second highest significant result was achieved by NAA at 1.0 mg/L. In both control and 5.0 mg/L of auxins produced the nethermost root number as compared to others. The roots were weak, further growth was not satisfactory, the leaves gradually turned pale yellow, abscised and ultimately the plants were not survived. It was also observed that NAA at 2.0 mg/L formed much soft white callus in the basal portion of root which encumber in root formation and elongation.

Among the various auxins, NAA at 1.5 mg/L significantly produced the maximum number of 7.5 roots suggesting its higher potentiality to initiate active roots than other auxins. Further, IBA positioned the second next to IAA in respect to root formation. Kabir (2005) also reported a high incidence of root formation in stevia with NAA than IBA and IAA. A high concentration (5.0 mg/L) of auxins, the number and length of roots were significantly decreased and the minimum (1.3) roots were observed. Such trend of reduction in number and length of roots by high concentration of auxins might be due to the enhancement of ethylene biosynthesis in the root tissue (Waring and Phillips, 1981).

Table2. Effect of IBA, NAA and IAA on rooting of stevia.

Treatments (mg/L)		% of cutting rooted	No. of root/ rooted cutting
IBA	0.5	50	3.8
	1.0	70	4.6
	1.5	80	5.7
	2.0	40	4.0
	5.0	30	1.3
NAA	0.5	60	4.3
	1.0	90	6.0
	1.5	100	7.5
	2.0	80	5.5
	5.0	30	1.5
IAA	0.5	50	3.5
	1.0	50	4.5
	1.5	60	5.0
	2.0	40	4.2
	5.0	30	1.4
Control		20	1.0
CV (%)		8.65	11.29

There were 3 replication in each treatment and data were recorded after 8 weeks of culture.

Plate 6. Roots produced with ½ MS + NAA at 1.5 mg/L after 8 weeks of culture.

Experiment 4: Effects of 2, 4-D and different explants on callus initiation.

In this experiment, 1.0, 2.0, 3.0, 4.0, 5.0, and 6.0 mg/L of 2, 4-D and young leaf disk, mature leaf disk and stem node were used to observe their effect on callus initiation.

Percentage of callus initiation.

The results on the main effect of explants and different concentrations of 2-4-D on percentage of callus initiation have been presented in table 3. Significant influence was found on callus initiation from different explants. At 20 DAI, young leaf disc showed the height percentage of callusing (50.00%) followed by stem node (33.33%) at 3.0 mg/L 2-4-D. But the mature leaf disc showed no callus initiation out of six cultured explants.

Table 3:Effect of different concentration of 2,4-D in MS medium on percentage of callus initiation from young leaf disc, older leaf disc and node of stem of stevia. Data were recorded at 20, 30 and 40 DAI. Six explants were placed in each treatment (with 4 replication)

Treatment		% of callus initiation		
Explant	**Hormone**	**20 DAI**	**30 DAI**	**40 DAI**
Young leaf	0	0.00	0.00	0.00
	1	0.00	16.66	33.33
	2	16.66	33.33	50.00
	3	50.00	66.66	100.00
	4	16.66	33.33	50.00
	5	16.66	16.66	33.33
	6	0.00	16.66	33.33
Old leaf	0	0.00	0.00	0.00
	1	0.00	0.00	0.00
	2	0.00	16.66	16.66
	3	0.00	16.66	16.66
	4	0.00	16.66	16.66
	5	0.00	0.00	16.66
	6	0.00	0.00	0.00
Stem node	0	0.00	0.00	0.00
	1	0.00	0.00	33.33
	2	16.66	16.66	33.33
	3	33.33	50.00	66.66
	4	16.66	16.66	33.33
	5	0.00	16.66	33.33
	6	0.00	0.00	0.00
LSD (0.01)		17.67	22.21	30.68

At 30 and 40 DAI, similar trend of results was found where the highest percentage of responsive calli was observed from young leaf disc (66.66% and 100% at 30 DAI and 40 DAI respectively.) followed by stem node (50% and 66.66% at 30 DAI and 40 DAI respectively). At the same time the lowest percentage of responsive calli (16.66% at 30 DAI and 40 DAI) was found with mature leaf disc.

The results indicated that the percentage of callus initiation was increased with the increase in concentration of 2,4-D up to a certain limit. Higher concentration was not good for callus initiation. Always moderate amount showed good result for all types of explants. In this experiment, 3 mg/L of 2,4-D showed the highest percentage young leaf disc in callus initiation. Stewart and Button (1978) showed that a range of 0.5-3.0 mg/L was beneficial and higher level was lethal for callus production. This result supports the present investigation. Stem node also showed moderate results in callus initiation, whereas mature leaf disc showed poor performance. Cooke (1977) also found that mature leaves from mature plants cultured on modified MS medium failed to proliferate which was also supported by Vij and Kaur (1999) who said that the success of foliar explants for *Ascocenda* was dependent upon the juvenility of tissue on callus initiation.

Morphogenic response of callus

During callus initiation, firstly leaf disks were swelled, and then formed water soaked to yellowish green callus. Callus started appearing at one end of explant within 4 weeks of inoculation in all concentrations of 2, 4-D. In next 3 weeks, rapid proliferation of callus was achieved at 1.0, 2.0 and 3.0 mg/L of 2, 4-D while at 4.0, 5.0 and 6.0 mg/L; it remained confined to one end only. After 7 weeks, entire explants were seen to be covered with green, globular and compact callus at 3.0 mg/L 2, 4-D. The callus formed with other treatments was yellowish green and less compact. Thus, 3.0 mg/L of 2, 4-D was proved best for obtaining good quality callus in stevia.

Table 4. Effect of 2, 4-D in MS medium on morphogenic responses of callus induction from young leaf disk of stevia after 7 weeks of culture

Treatments (2, 4-D)	Nature and amount of callus from young leaf disc
Control	-
1.0 mg/L	Light green, compact, ++ extending moderately over the explants
2.0 mg/L	Light green, semicompact ++ extending moderately all over the explants
3.0 mg/L	Green, compact, +++ extending all over the explants
4.0 mg/L	Yellowish green, semi compact + extending one end only
5.0 mg/L	Yellowish green, compact, + extending one end only
6.0 mg/L	Yellowish green, compact, + extending one end only

Relative amount of Callus

= Nil; + = Poor; ++ = Moderate; +++=Extensive

Plate 7. Callus induction from young leaf disc explant of stevia at 3.0 mg/L 2, 4-D

Experiment 5. Effects of NAA and BAP on growth and Development of regenerated plantlets.

Callus (0.3g) cultured on MS basal medium supplemented with different concentration and combination of NAA and BAP {T_1-control, T_2-1.0 mg/L BAP+0.5 mg/L NAA, T_3-1.0 mg/L BAP + 1.0 mg/L NAA, T_4- 1.0 mg/L BAP+ 2.0 mg/L NAA, T_5-2.0 mg/L BAP+ 1.0 mg/L NAA, T_6-2.0 mg/L BAP+ 2.0 mg/L NAA} to observe their effect on the subsequent growth and development of initiated stevia plantlets. Data were recorded at 30, 60 and 90 days after inoculation (DAI). The results of the present investigation have been presented in Figures 3 and 4.

Number of proliferated shoots

At 30 DAI, the highest shoot number (4.5) was found with 1.0 mg/L of BAP + 0.5 mg/L of NAA (T_2). The least number of shoot was obtained with control (T_1). The figure 3 revealed that number of shoot was increased gradually at different DAI. From these results, it was found that T_2 helped in increasing the number of shoots. However, in this experiment, 1.0 mg/L of BAP + 0.5 mg/L of NAA (T_2) gave the best result (8.9 shoots/ explant) in respect of highest shoot proliferation. But further increase in the concentration of BAP and NAA (T_3-T_5) had decreased effect on number of shoot in stevia. This result is in support with the result of Acuna *et al.*, (1997) who observed that the highest frequency of shoot regeneration (91.5%) and the highest number of shoot formation (10 shoots/explant) in stevia were recorded with 1.0 mg/L of BAP with 0.5mg/L NAA. Rida *et al.*, (1999) also supported that medium with 1.0 mg/L BAP and 0.5 mg/L NAA gave the best yield of 13 shoots/explant in dianthus after 15 weeks.

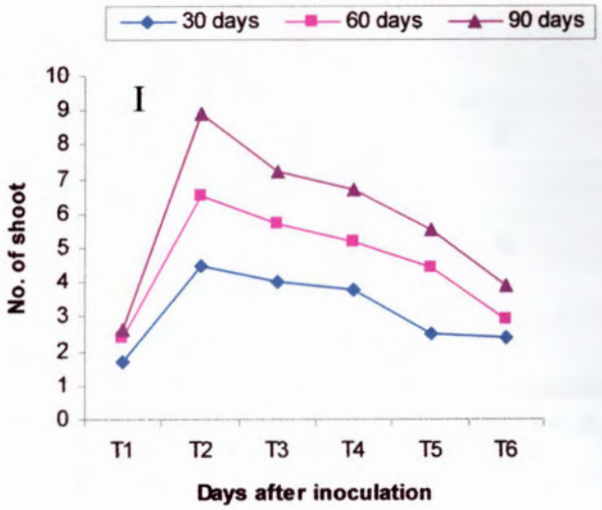


Fig. 3. Effect of different concentration and combination of NAA and BAP for shoot proliferation in stevia from calllus. Vertical bar represent LSD value at 1% level of probability.

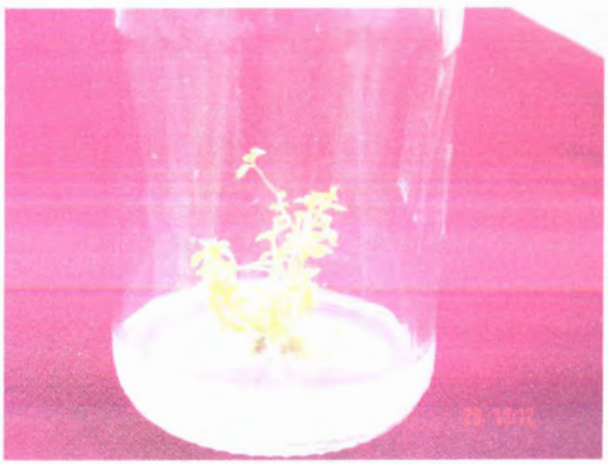


Plate 8(a). Multiple shoot of stevia from callus

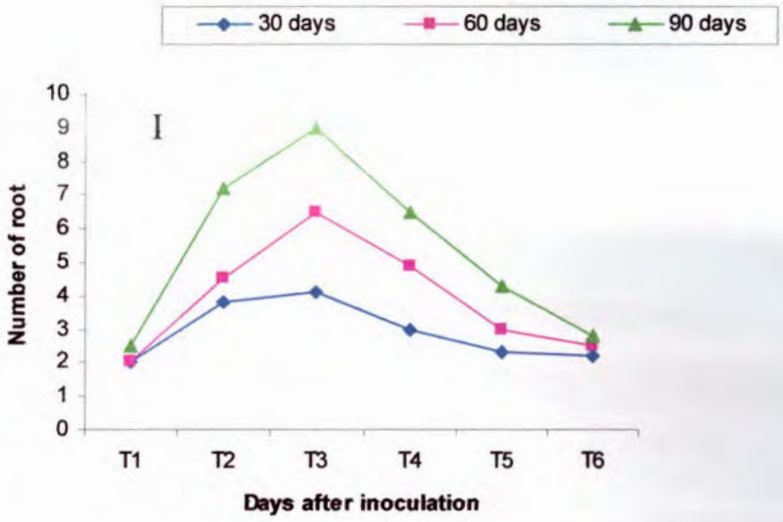


Fig. 4. Effect of different concentration and combination of NAA and BAP for root induction in stevia. Vertical bar represent LSD value at 1% level of probability.

Number of roots

The figure 4 revealed that number of root was increased gradually at different DAI. The least number of roots was observed in control. In this experiment, it was found that the number of roots increased with the increase of concentration of NAA and BAP up to a certain limit. However the highest number of root formation (9 roots /shoot) in stevia were recorded with T_3 (1.0 mg/L of BAP with 1.0mg/L NAA) followed by T_2 (1.0 mg/L of BAP with 0.5mg/L NAA) This result is supported with the findings of Panthania *et al.*, (1998) who found that the1/2 MS media favoured rooting when supplemented with 1.0 mg/L of BAP +1.0mg/L NAA.

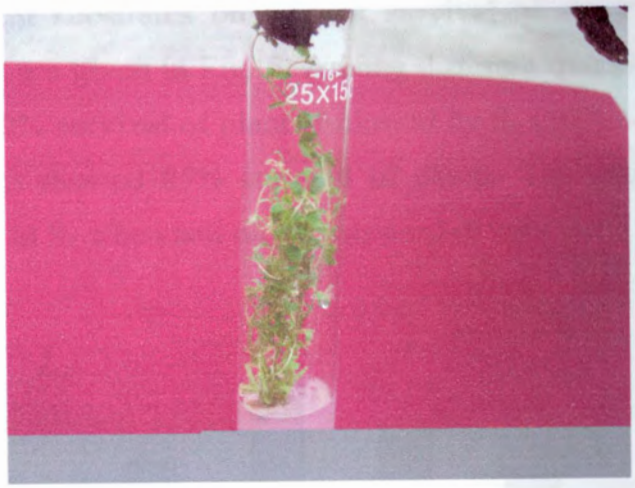


Plate 8(b). Shoot proliferation and elongation of stevia

Plate 9. Root formation and elongation of stevia

Experiment 6: Influence of different substrates on *ex vitro* establishment of stevia

The effect of different substrates on percent survival of stevia plants raised through tissue culture are given in Fig. 5. Among different treatments, S_3 (100% cocodust) showed 100% survival of plants followed by S_4 (50% cocodust + 25% soil + 25% cowdung) showed 85% survival of plants. The lowest survivality percentage was noted in S_1 when soil alone was used as substrate

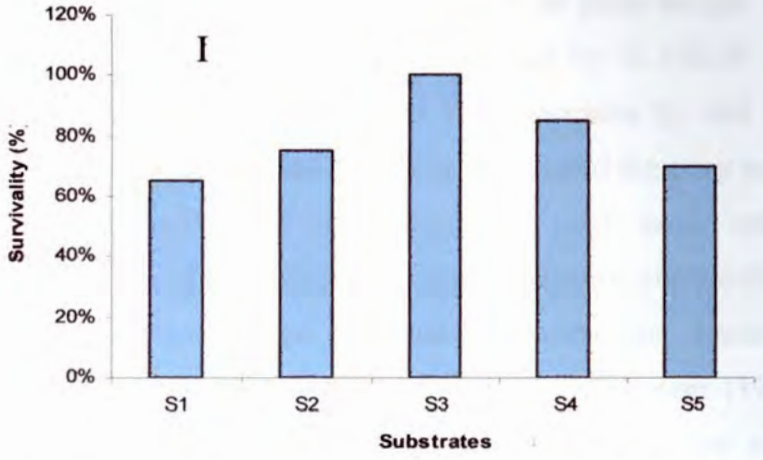


Fig. 5. Effect of substrates on *ex vitro* establishment in stevia. Vertical bar represent LSD value at 1% level of probability.

Table 5. Effect of substrates on some morphology and growth characteristics in *ex vitro* establishment of stevia (Data were recorded after 3 months)

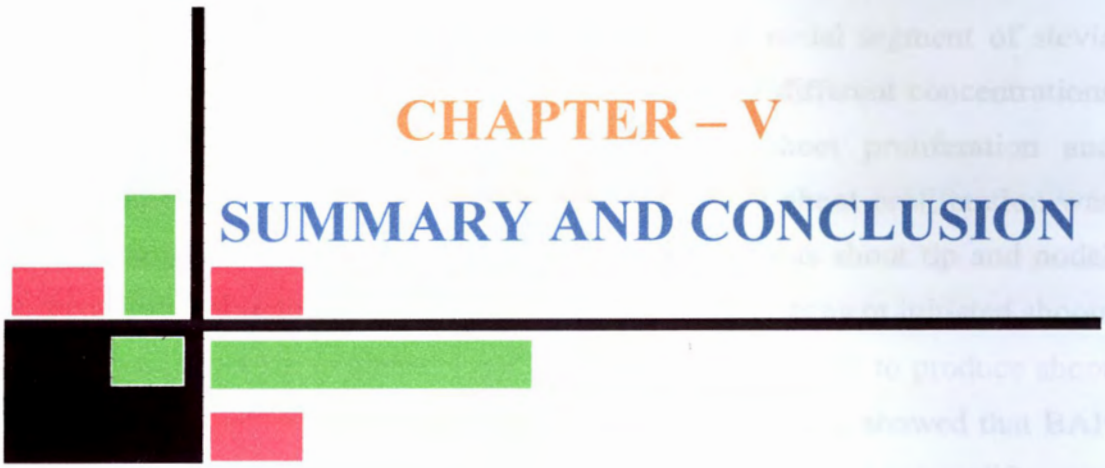
Type of Substrate	No. of leaf/ plantlet	No. of shoot/ plantlet	Plant spread (cm)	Plant height (cm)	Growth response
S_1	20.00 d	4.00 c	5.00c	7.00 c	-
S_2	24.00 cd	7.00 bc	8.00 b	11.00 bc	+
S_3	49.00a	12.00 a	15.00 a	19.00a	++
S_4	37.00b	10.00 ab	13.00 ab	18.00 a	++
S_5	28.00c	8.00 b	13.00 ab	15.00 ab	+

Means bearing uncommon letter (s) in a column varied significantly at 5% level.

It revealed from the Table 5 that all the parameters related to *ex vitro* performance of stevia significantly influenced by different substrates. Among different substrates studied, highest number of leaf (49.00) and shoot (12.00) was found with the substrate contained 100% cocodust (S_3) the moderate number of leaf (37.00) and shoot (10.00) was observed by 50% cocodust with 25% soil and 25% cowdung (S_4) while the minimum number of leave (20.00) and shoot (4.00) was noticed when the substrate was only soil (S_1). Maximum plant spread (15.00 cm) was observed at S_3 closely followed by S_4 (13.00 cm) and S_5 (13.00 cm). Substrates soil (S_1) responded poorly (5.00 cm) in regard of attaining a considerable plant spread in stevia. In respect of plant height, the longest plant was observed at S_3 (19.00 cm) closely followed by S_4 (18.00 cm). The overall plant growth responses were excellent for substrates S_3 and S_4. The medium response was observed in S_2 and S_5, while S_1 showed the poor response.

In carnation plant grew well in a mixture of peat: sand: oan (1: 1:1:1) v/v) covered with a layer of peat and sand then perlite or vermiculite mixes (Stone, 1963). There are reports that all rooted plants after hardening were well established in the soil (Hoque and Fatema, 1995). Islam (1999) successfully established chrysanthemum plantlets in soil: sand: rice burn ash at 1:1:1 ratio. Ara (1997) reported that rose plantlets successfully grew well in 100% vermiculite. However, in the present study 100% cocodust performed best for *ex vitro* establishment. Actually the nature and types of substrate varies from species to species. The better performance of 100% cocodust for *ex vitro* establishment in stevia might be good water holding capacity, good aeration system and proper nutrient status.

Plate 10. *Ex vitro* establishment of stevia



CHAPTER – V

SUMMARY AND CONCLUSION

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Summary

An investigation was carried out with stevia for *in vitro* propagation at Biotechnology Laboratory, Bangladesh Sugarcane Research Institute (BSRI), Ishurdi, Pabna, during the period of January-October/2006. Shoot tip, leaf base and nodal segments were used as explants for shoot regeneration of direct *in vitro* propagation. Only leaf disk was used for indirect *in vitro* propagation through callus culture. All the explants were collected from 6-8 months old yard grown plants at Biotechnology Laboratory, BSRI.

The explants, shoot tip, leaf base with petiole and nodal segment of stevia were cultured on MS medium supplemented with 5 different concentrations (0.0,1.0,2.0,3.0,4.0 and 5.0 mg/L) of BAP for shoot proliferation and elongation of stevia. The present study revealed that shoot proliferation was influenced by the type of explants. Among the explants shoot tip and nodal segment were able to produce multiple shoots. Nodal segment initiated shoots earlier than shoot tip explants. Leaf base with petiole failed to produce shoot on any of the concentration of BAP used. The study again showed that BAP concentration of 3.0 mg/L exhibited the best response for shoot proliferation and elongation.

The experiment with various cytokinin showed that BAP applied alone was the most effective cytokinin for stevia. When BAP was used in combination with 2ip and Kn, the number of shoot and their length were decreased .It was also observed that both KnN and 2ip either alone or in combination was not significant over BAP alone. However, the highest shoot number (8.0) and length (7.8 cm) was found with the application of BAP 3.0 mg/L. Multiple shoots were also obtained in control treatment and the highest concentration (5.0 mg/L) of all cytokinin but in both concentrations, elongation of shoots was poor, weak in nature and ultimately they turned green to brown in colour.

Excised shoots collected from *in vitro* grown materials and were cultured into

½ MS media having 4 concentrations of IBA, IAA and NAA ranging from 0.5 to 5.0 mg/L. The experiment included a control which received no auxin. The result revealed that NAA at 1.5 mg/L produced significantly higher number of roots than all other auxin concentrations. Each shoot produced 7.5 roots within 8 weeks of incubation in treatment with 1.5 mg/L NAA. The second highest significant result was achieved by NAA at 1.0 mg/L. Out of all auxin concentrations tested, in both control and 5.0 mg/L produced the nethermost root number as compared to others. The roots were weak, root colour turned black instead of remaining white, further growth was not satisfactory, the leaves gradually turned pale yellow, abscised and ultimately the plants were not survived.

The plantlet initiation via callus is a possible method for rapid and economical production of stevia and it has the potential for rapid mass clonal production also. Callus initiation was observed on MS medium containing different concentrations viz.0.0, 1.0, 2.0, 3.0, 4.0, 5.0 and 6.0 mg/L 2,4-D and different explants viz. young leaf disc, mature leaf disc and stem node on callus initiation and morphogenic response of callus. The percentage of calli initiation was the highest incase of young leaf disc (100%) at 3.0 mg/L 2,4-D after 40 DAI. Stem node showed moderate result in percentage of callus initiation (50 %) at 3.0 mg/L 2,4- D after 40 DAI. The mature leaf disc showed very poor response in callus initiation. Among three explants, young leaf disc at 3.0 mg/L 2,4-D showed the best performance in calli induction whereas no calli were formed with control. During callus initiation, firstly leaf disks were swelled, and then formed water soaked to yellowish green callus. Callus started appearing at one end of explant within 4 weeks of inoculation in all concentrations of 2, 4-D. In next 3 weeks, rapid proliferation of callus was achieved at 1.0, 2.0 and 3.0 mg/L of 2, 4-D while at 4.0, 5.0 and 6.0 mg/L; it remained confined to one end only. After 7 weeks, entire explants were seen to be covered with green, globular and compact callus at 3.0 mg/L 2, 4-D. The callus formed with other treatments was yellowish green and fewer compacts. Thus, 3.0 mg/L of 2, 4-D was proved best for obtaining good quality callus in stevia.

Callus (0.3g) explanted on MS basal medium supplemented with different concentration and combination of NAA and BAP (T_1-control, T_2-1.0 mg/L BAP+0.5

mg/L NAA, T_3-1.0 mg/L BAP + 1.0 mg/L NAA, T_4- 1.0 mg/L BAP+ 2.0 mg/L NAA, T_5-2.0 mg/L BAP+ 1.0 mg/L NAA, T_6-2.0 mg/L BAP+ 2.0 mg/L NAA) to observe their effect on the subsequent growth and development of initiated stevia plantlets at 30, 60 and 90 days after inoculation (DAI). However, in this experiment, 1.0 mg/L of BAP + 0.5 mg/L of NAA (T_2) gave the best result (8.9 shoots/ explant) in respect of highest shoot proliferation after 90 days inoculation (DAI). But further increase in the concentration of BAP and NAA (T_3-T_5) had decreased effect on number of shoot in stevia. The root number was increased with the increase of concentration of NAA and BAP up to a certain limit. However the highest number of root formation (9 roots/ shoot) in stevia were recorded with T_3 (1.0 mg/L of BAP with 1.0 mg/L NAA) followed by T_2 (1.0 mg/L of BAP with 0.5 mg/L NAA).

Establishment of cultured plantlets depends on the development of suitable conditions to harden the plants rapidly. Stevia requires a suitable media with good physical and chemical conditions for their proper growth and development. The effect of different substrates on establishment of stevia plants raised through tissue culture were standardized. Among different treatments, S_3 (100% cocodust) showed 100% survival of plants. The study also indicated that highest plant height; maximum number of leaf as well as best growth response were also attained in this treatment. The better performance of cocodust might be due to high water holding capacity, good aeration and cation exchange capacity of the media So, cocofibre was found best for *ex vitro* plantlet establishment in stevia.

Conclusion

Nodal segments were produced more multiple shoots than shoot tip whereas leaf base with petiole failed to produce shoot on any of the concentration of BAP used. The study again showed that BAP concentration of 3.0 mg/L exhibited the best response for shoot proliferation and elongation.

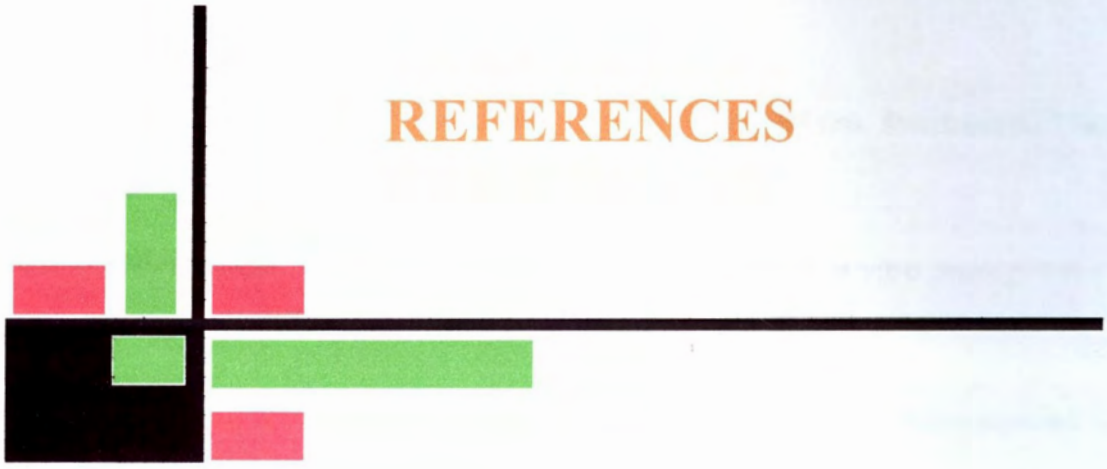
NAA at 1.5 mg/L produced significantly higher number of roots (7.5) than all other auxin concentrations.

Young leaf disc at 3.0 mg/L 2, 4-D showed the best performance in calli induction whereas no calli were formed with control.

1.0 mg/L of BAP + 0.5 mg/L of NAA (T_2) gave the best result (8.9 shoots/ explant) in respect of highest shoot proliferation whereas the highest number of root formation (9 roots /shoot) in stevia were recorded with T_3 (1.0 mg/L of BAP with 1.0 mg/L NAA) from callus explants.

The regenerated shoots with developed roots were successfully established into the pots after proper hardening. However, 100% cocodust medium was the best substrate for *ex vitro* establishment of stevia.

REFERENCES



REFERENCES

Abbott, A.J. 1978. Practice and promise of micropropagation of woody specices. *Acta Hort.* 79:113-128.

Acuna, I., A. Nepovim and P. Valicek. 1997 Micropropagation of plants *Stevia rebaudiana* Bertoni *in vitro* and content of stevioside in leaves after application of growth regulators under field conditions. *Agricultura Tropica et Subtropica.* 30: 51-60.

Alam, S. 2003. *In vitro* embryo culture and plantlet regeneration of *Phaius tankervillae.* An M. S. Thesis submitted to the Department of Horticulture, BAU, Mymensingh, Bangladesh. Pp.39-60.

Anderson, W.C. 1978. Rooting of tissue cultured rhododendrons. International Plant Propagators Society, Combine Proceeding. 28:135-139.

Ara, A., M. I. Hoque, R.H. Sarker and M. M. Haque. 1991a. *In vitro* propagation of *Sesbainia grandiflora* L. Plant Tissue Cult. 1 (2): 79-84.

Ara, A., M. T. Hoque, R. H. Sarker and M.M. Haque. 1991b. Micropropagation of *Leucaena leucocephala* L. Plant Tissue Cult. 1 (2): 115-119.

Ara, K. A. 1997. *In vitro* propagation of rose (*Rosa sp.*). An M. S. Thesis submitted to the Department of Horticulture, IPSA, Salna, Gazipur, Bangladesh. pp. 45-50.

Atkins, C. 1994. Stevia . Health Revelations Newsletter. 2(1): 32-34.

Barve, D.M., R.S. lyer, S. Kendurkar and A.F. Mascarenhas . 1984. An effective method for rapid propagation of some budded rose varieties. India J. Hort. 41: 1-7.

Begum. S. and S. Hadiuzzaman. 1995. *In vitro* rapid shoot proliferation and corm development in *Gladiolus grandiflourus* cv. Red brand. Plant Tissue Cult.5

(1): 7-12.

Bhattacharjee, S. K. 1985. Cultural management in orchids. [In: Progress in Orchid Research. (Eds.) Chadha M. L. and F. Singh.] I. I. H. R. Bangalore. pp. 25-55.

Bhattacharjee, S.K. and T. Mukherjee. 1981. Effect of potting media on growth and flowering of some epiphytic and terrestrial orchids. Haryana J. Hort. Sci. 10 (1, 2): 58-66.

Bhosle, S. 2004. Commercia Cultivation of *Stevia rebaudiana*, Agrobios Newsletter. 3 (2): 43-45.

Biswas, B., R. Bhattacharjee and J. Biswas. 1994. Effect of different potting media on growth and flowering of certain epiphytic and terrestrial orchids. Souvenir and Abstracts. National seminar on a decade of orchid research and development. The Orchid Soc. India. p. 90.

Bonga, J.M. 1977. Applications of tissue culture in forestry. In: Applied and fundamental aspects of plant cell, tissue and organ culture. J. Reinert, Y.P.S. Bajaj (eds), pp 93-108. Springer-Verlag, Berlin.

Brandle, J.E. and N. Rosa. 1992. Heritability for yield, leaf-stem ratio and stevioside content estimated from a landrace cultivar of *Stevia rebaudiana*. Canadian Journal of Plant Science. 72: 1263-1266.

Bressen, P.H., Y.J. Kim, S.E. Hyndman, P.M. Hasegawa and R.A. Bressen. 1982. Factors affecting *in vitro* propagation of rose. J. Am. Soc. Hort. Sci. 107:979-990.

Carneiro, J.W.P. 1990. *Stevia rebaudiana (Bert.) Bertoni*, production of seed. An M.Sc Thesis submitted to the State University of Maringa, Brazil.

Chang, C. and W. C. Chang. 1997. Plant regeneration from callus culture of *Cymbidium ensifolium* var misericors. Plant Cell Rep.17 (4): 251-255.

Chen, J.T., W.C. Chang, J.T. Chen and W.C. Chang. 2000. Efficient plant regeneration through somatic embryogenesis from callus cultures of *Oncidium* (Orchidaceae). Plant Sci. 160(2): 97-103.

Chowdhary, M.L. 1991. Vegetative propagation of rose *in vitro* through multiple shoot development. India J. Hort. 48(2): 177- 181.

Cooke, R.C. 1977. Tissue Culture Propagation of African violets. Hort. Sci. 12 (6): 549.

Crammer, B. and R. Ikan. 1986. Sweet glycosides from the stevia plant. Chemistry in Britain .22: 915-916.

Dantu, P.K. and S.S. Bhojwari. 1987. *In vitro* propagation and corm formation in Gladiolus. Gartenbauwissenschalft. 52(2) S: 90-93.

Davies, D.R. 1980. Rapid propagation of rose *in vitro*. Sci. Hort. 13:385-389.

Debergh, P.C. and R.E. Read. 1990. Micro propagation. In: Micro propagation: Technology and Application. Deborah, P.C. and R.H. Zimmerman, (Eds). Kluwer Academic Publ. Dordrecht, The Netherlands, pp 1-12.

Duke, J. 1993. *Stevia rebaudiana*. Handbook of alternative cash crops. CRC Press, Boca Raton, FL. pp 422-424.

Elliot, R.F. 1970. Auxinic culture of meristem tips of Rosa multiflora. Planta. 16:490.

Ellyard, R.K. 1981. Rooting hormones: Their effeft on the rooting of some Austration species. Aust. plants. 11: 161-165.

Evans, P. K., N. Haq and S. Cooper-Bland. 1981. Tissue Culture of the winged bean. A paper submitted on the proceedings of International workshop on Improvement of Tropical Crops Through Tissue Culture held on March 9-14. p. 107-111.

Fonnesbech, A. and M. Fonnesbech.1979. *In vitro* propagation of *Monestera deliciosa*. Hort. Sci. 15:740-741.

Fors, A. 1995. A new character in the sweetener scenario. Sugar J. 58: 30.

Fridborg, G. and T. Eriksson. 1975. Effect of activated charcoal on growth and morphogenesis in cell cultures. Physiol. Plant.34: 306-308.

George, L. and S. Eapen. 1999. Thidiazuron induced shoot organogenesis and plant regeneration in chickpea (*Cicer arietinum* L.). Plant Tissue Cult. 9 (1): 71-78.

Goenadi, D. H. 1983. Water tension and fertilization of *Stevia rebaudiana (Bert.)* Menara Perkebunan .51: 85 – 90.

Goyal, Y., R.L. Bingham and P. Felker. 1985. Propagation of tropical tree, *Leucaena leucocephala* K67 by *in vitro* bud culture. Plant Cell Tissue Organ Cult. 4:3-10.

Grewal, M.S., J.S. Arora and S.S. Gosal. 1995. Micro-propagation of Gladiolus through *in vitro* cormlets production. Plant Tissue Cult.5 (1): 27-33.

Hakim, L., S.N. Hossain, S. Rahman, and M. Hossain. 1995. *In vitro* regeneration of plants from axillary bud cultures of *Ficus religiosa*. Plant Tissue Cult. 5(2): 131-135.

Hasegawa, P.M. 1979. *In vitro* propagation of rose tissue culture. Hort. Science. 14:610-612.

Hoque, M. I., T. J. Mustari and R. H. Sarker. 1996. *In vitro* shoot regeneration and *ex vitro* rooting in *Chrysanthemum morifolium* Ramat Plant Tissue Cult. 8 (2): 157-164.

Hoque, M.I. and M. Fatema. 1995 *In vitro* multiple shoot regeneration in *Chrysanthemum morifolium* Ramat. Plant Tissue Cult.5 (2): 153-162.

Huda, N. 2006. *In vitro* morphogenic responses of different explants of Stevia (*Stevia rebaudiana*). An M. S. Thesis submitted to the Discipline of Biotechnology

and Genetic Engeneering, Khulna University, Khulna, Bangladesh. pp.24.

Islam, M. O. 1999. Callus culture and plantlet regeneration in Doritaenopsis. J. Japanese Soc. Hort. Sci. 68(6): 1124-1131.

Islam, R., M. R. Karim, M. Hossain and O. I. Joarder. 1992. Plant Regeneration from leaf-derived callus in *Aegle marmelos* corr. Plant Tissue Cult. 2 (2): 55-59.

Islam, T. 2004. *In vitro* propagation of Stevia (*Stevia rebaudiana*). An M. S. Thesis submitted to the Discipline of Biotechnology and Genetic Engeneering, Khulna University, Khulna, Bangladesh. pp. 51.

Jain, M. and H.C. Chaturvedi. 1993. *In vitro* mass multiplication of *Chrysanthemum cinerarifolium*, in proc, XVI Annual Meeting of plant Tissue culture Association India and Symp. on plant tissue culture and its biotechnological applications. Mar. 1-3, 1993. Vniv. Rajashtal, Jaipur, India. pp. 9-10.

Jones, O.P. 1983. *In vitro* propagation of tree crops. In: Plant biotechnology. S.H. Mantell and H. Smith (eds). pp. 139-159. Cambridge University press, Cambridge.

Kabir, 2005. *In vitro* propagation and utilization of Stevia (*Stevia rebaudiana*). An M. S. Thesis submitted to the Department of Biotechnology, BAU, Mymensingh, Bangladesh. pp. 65.

Kamo, K., J. Chen and R. Lawson. 1990. The establishment of cell suspension cultures of gladiolus that regenerated plants *in vitro* cells. Dev. Biol. 26: 426-430.

Kashem, A.1992. *In vitro* Regenerative Performance of Seven *Cultivars of Gladiolus grandiflorus* L. M.Sc. thesis submitted to the Department of Botany, University of Dhaka. Bangladesh. pp.1-116.

Katayma, O., T. Sumida, H. Hayashi and H. M. Itsuhashi. 1976. The practical

application of Stevia and research and development data (English translation). I.S.U. Company, Japan. pp. 747.

Khosh-Khui, M. and K.C. Sink. 1982. Rooting enhancement of Rosa hubrida for tissue culture propagation. *Scientia Hort*. 17: 371-376.

Kinghorn, A.D. 1992. Food ingredient safety review: *Stevia rebaudiana* leaves. Herb Research Foundation, Boulder. pp. 10-13.

Kinghorn, A.D. and D.D. Soejarto. 1985. Current status of stevioside as a sweetening agent for human use. In Wagner, Hikino, H., and Fransworth, N.R. (eds). Economic and medical plant research. Academic Press. London.

Kyte, L. 1987. Plants from test tube. Timber press. Oregon. p. 133.

Lee, J. I., K.K. Kang and E.U. Lee. 1979. Studies on new sweetening resource plant Stevia (*Stevia rebaudiana* Bert.) in Koprea. I.Effect of transplanting date shifting by cutting and seeding dates in agronomic characteristics and dry leaf of Stevia (English abstr) Res. Rep. ORD, 21:171-179.

Lewis, W.H. 1992 Early uses of *Stevia rebaudiana* (Bert) leaves as a sweetener in Paraguaya. Econ. Bot, 46:336-337.

Metivier, J. and A.M. Viana .1979. The effect of long and short day length upon the growth of whole plants and the level of soluble proteins, sugar and stevioside in leaves of *Stevia rebaudiana* Bert. J. Exp. Bot. 30: 1211-1222.

Miyagawa, H., N. Fujikowa, H. Kohda, K. Yamasaki, K. Taniguchi and R. Tanaka. 1986. Studies on the tissue culture of *Stevia rebaudiana* and its components. Planta Medica. 4: 321-324.

Morel, G. and J.F. Muller. 1964. La culture *in vitro* de meristem apical de la pomme

de terre. Compl. Rend. Acad. Sci. Paris. 258:5250-5252.

Morita, T. 1987. Dried leaves of Stevia. Jap. Patent.63- 96.

Murai, N. 1988. Stevia drying system. Jap. Patent. 65 –81.

Murashige, T. 1974. Plant propagation through tissue culture. Ann. Rev. Plant physiol. 25: 135-166.

Murashige, T. 1978. The impact of tissue culture in agriculture. In: T. Thorpe, (Ed.). Frontiers of Plant Tissue Culture, University of calgary Press. Canada, pp.15-28.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bio-assays with tobacco cultures. Physiol. Plantarum.15: 473-497.

Nayak, N.R., P.K. Chand, S.P. Rath and S.N. Patnaik. 1998. Influence of some plant growth regulators on the growth and organogenesis of *Cymbidium aloifolium.In vitro* Cell. Dev. Biol. Sci. 34(3): 185-188.

Nidhi, P. 1998. Effect of thidiazuron and BAP on shoot buds differentiation from excised cotyledons of *Brassica juncea* (L) Czem. Plant Tissue Cult. 8 (1): 55-60.

Nitsch, J.P. and C. Nitsch. 1969. Haploid plants from pollen grains. Science .163:85-87.

Pathania, N.S., O.P. Sehgal, P. Debojit, B.S. Dilta and D. Paul.1998. Studies on micropropagation in *Dendrobium* cv. Sonia. J. Orchid Soc. India. 12(2): 35-38.

Puite, K.J. 1992. Globular somatic embryos on leaf of *Stevia rebaudiana.* Plantarum. 85: 403-410.

Rao, A. V. N. 1985. Endangered Paphiopedilum. [In: Progress in Orchid Research. (Eds.) Chadha and Singh.] I. I. H. R. Bangalore. pp. 62-69.

Rida, A. S., M. M. Ajlouni, M. A. Shatnawi and A. Abu-Ein. 1999. Shoot regeneration in Carnation. Plant Tissue Cult. 9 (2): 159-166.

Raju, B., K. T. Shivarhankar, G. K. Seetharmu and K. S. Sheshsgiri.1997. Micropropagation of Dendrobium. [In:Developmental Biology and Commercialization of Orchids (Ed.) S.P. Vij] Aff. East-west Pvt. Ltd. New Delhi. P. 86.

Salas, P. 2001. Use of stevia. Proceeding of 9th International Conference of Horticulture, September 3th- 6th 2001 Lednice, Czech Republic, ISBN 80-7157-524-0. 2: 431-434.

Sauer, A., F. Walther and W. Preil. 1985. Different suitability for *in vitro* propagation of rose cultivars. Gartenbauwiss. 50: 133-138.

Saxena, N. C. and L. S. Ming. 1988. Preliminary harvesting characteristics of Stevia. Phys. Prop. Agric. Mat. Prod. 3: 299 – 303.

Shaffert, E.E. and A.A. Chetober. 1994a. Development of the female gametophyte in *Stevia rebaudiana* (English translation). Buletinul Academiei de Schtintse.A Republica Muldava. 6: 10-18.

Shaffert, E.E. and A.A. Chetober. 1994b. Structure, topography and ontogeny of *Stevia rebaudiana* (English abstr.). Botanicheskii Zhurnal .79: 38-48.

Shizhen, S. 1995. A study on good variety selection in *Stevia rebaudiana*. Scientia Agricultura Sinica. 28: 37 – 41.

Shock, C.C. 1982. Experimental cultivation of Rebaudia's Stevia in California University of California .Agronomy Progress Report .No. 122.

Sivaram, L. and U. Mukundan. 2003. *In vitro* culture studies on *Stevia rebaudiana*. *In vitro* Cellular and Developmental Biology. Plant. 39 (5): 520–523.

Skirvin, R.M. and M.C. Chu. 1979. *In vitro* propagation of 'Forever Yours' rose. *Hort. Science*. 14:608-610.

So, I.S. 1983. Studies on *in vitro* Culture of *Sainpaulia ionantha* Wendl. Korean Soc. *Hort. Sci.* 24 (1): 86-91.

Soejarto, D.D., C.M. Compadre, P.J. Medon, S.K. Kamath and A.D. Kinghorn. 1983. Potential sweetening agents of plant origin. J. Nat. Prod. 45: 590-599.

Start, N.D. and B.G. Cumming. 1976. *In vitro* Propagation of *Sainpaulia ionantha* Wendl. *Hort. Sci.* 11 (3): 204-206.

Stewart, J. and J. Button. 1978. Development of callus and plantlets from *Epidendrum* root tips cultured *in vitro.* Amer. Orchid Soc. Bull. 47 (7): 607-612.

Stone, O.M. 1963. Factors affecting the growth of Carnation plants from shoot apices. Ann. Appl. Biol. 52: 199-209.

Strauss, S. 1995. The perfect sweetener. Technol. Rev. 98: 18 – 20.

Tamura, Y., S. Nakamura, H. Fukui and M. Tabata. 1984. Comparison of Stevia plants grown from seeds, cutting and stem tip cultures for growth and sweet deterpene glycosides. Plan Cell Rep. 3: 180-182.

Uddin, M.S. 1993. *In vitro* propagation of Thuja (*Thuja orientalis*) through shoot tip culture. An MS thesis. submitted to the Dept. of Hort. IPSA. pp. 36.

Valio, I.F.M. and R.F. Rocha. 1966. Effect of Photoperiod and growth regulators on growth and flowering of *Stevia rebaudiana* Bertoni. Jap. J. Crop Sci. 46: 243-248.

Van Hooren, D. L. and H. R. Lester. 1992. Stevia drying in small scale bulk tobacco kilns. Final report. Ontario Ministry of Agriculture and Food. Delhi.pp.140.

Venkatachalam, P., N. Geetha and A. Saravanababu. 1998. Rapid Multiplication of

Eucalyptus camaldulensis by *in vitro* culture of nodal explant. Plant Tissue Cult. 8 (2): 101-107.

Vij, S.P., K. Parmindor and P. Kaur. 1999. Rapid clonal multiplication of *Asscocenda* through *In vitro* culture of leaf explants. Proceedings of the National Academy of Sciences, India. Biol. Sci. 69 (3-4): 317-321.

Waring, P.F. and I.D.J. Phillips. 1981. Growth and differentiation in plants. 3rd edn. Pergamon press, U.K.

Whitaker, J. 1994. Stevia. Whitaker's Newsletter .pp.11-14.

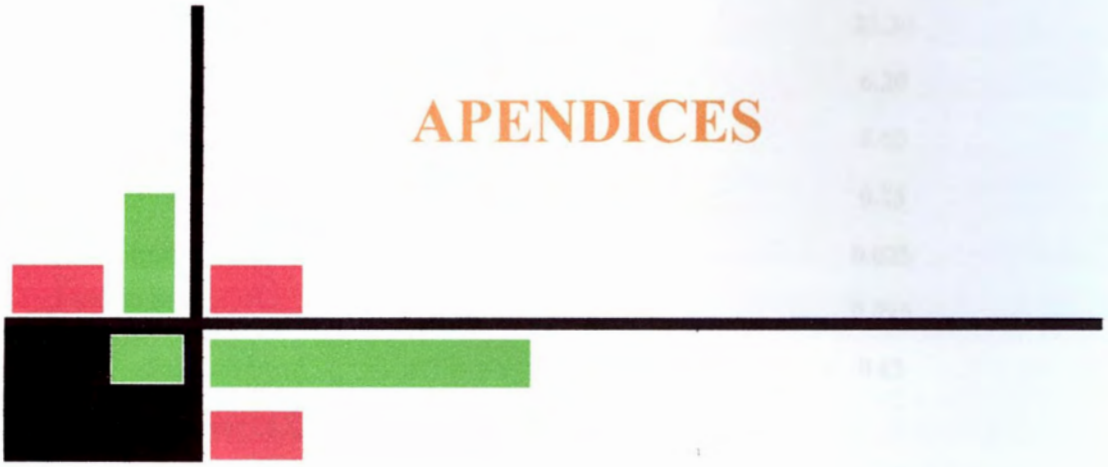
Whitehead, H.C.M. and K.L. Giles. 1976. Rapid propagation of poplars by tissue culture methods. International plant propagation Society, Combined proceedings, 26:340-343.

Wilfret, G.J. 1971. Shoot tip culture of Gladiolus: An evaluation of nutrient media of callus tissue development. Proc. Fla. State. Hort. Soc. 84:389-393.

Xili, L., B. C. Chenggjiani, X. Eryi, S. Reiming, W. Yuengming, S. Haodong and H. Zhiyan. 1992. Chronic oral toxicity and carcinogenicity study of Stevioside in rats. Food Chem. Tox. 30: 957 – 965.

Zaidan, L.B.P., S.M.C. Dietriech and G.M. Felippe. 1980. Effect of photoperiod on flowering and stevioside content in plants of *Stevia rebaudiana* Bertoni. Jap. J. Crop Sci. 49: 569-574.

Ziv, M. 1979. Transplanting Gladiolus plants propagated *in vitro*. Scientia Hort. 11: 257-260.



APENDICES

Appendix I. Murashige and Skoog (MS) medium, 1962

Components	Concentrations (g/l)
Macronutrients(10X)	
KNO_3	1.90
NH_4NO_3	1.65
$MgSO_4.7H_2O$	0.37
$CaCl_2.2H_2O$	0.44
KH_2PO_4	0.17
Micronutrients (100X)	
$MnSO_4.4H_2O$	22.30
H_3BO_3	6.20
$ZnSO_4.7H_2O$	8.60
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
KI	0.83
Iron Source(10X)	
$FeSO_4.7H_2O$	27.8
$Na_2EDTA.2H_2O$	37.3
Vitamins/Organic nutrients (100X)	
Nicotinic Acid	0.5
Pyridoxine HCl	0.5
Thiamine HCl	0.1
Glycine	2.0
Myo inositol	100 mg
Sucrose	30 g
Agar	8 g

pH adjusted to 5.8 before autoclaving

Appendix II. Analysis of variance of the data on percentage of cutting rooted and number of root formation from the effect of different concentration of IBA, NAA and IAA

Sources of variation	Degrees of freedo	Mean square	
		% of cutting rooted	No. of root
Replication	2	212.250	1.577
Treatment	15	1680.00**	10.544**
Eroor	30	22.650	0.203

** = Significant at 1% level of probability

Appendix III. Mean square of analysis of variance of the data on percentage of callus initiation.

Source of variation	Degrees of freedom	Mean square of percentage of callus initiation		
		20 DAI	30 DAI	40 DAI
Factor A (2,4-D)	6	209.804^{*}	885.516^{**}	1498.325^{**}
Factor B (Explant)	2	711.449^{**}	5532.361^{**}	12492.351^{**}
AB	12	145.128^{NS}	235.802^{NS}	308.260^{NS}
Error	63	88.483	139.892	16805.926

* = Significant at 5% level of probability
** = Significant at 1% level of probability
NS = Non significant
DAI = Days After Inoculation

Appendix IV. Mean square of analysis of variance of the data on growth characteristics in ex vitro establishment of stevia after three months of hardening.

Source of variation	Degrees of freedom	Mean square			
		No. of leaf	No. of shoot	Plant spread	Plant height
Replication	2	5.60 ^{NS}	7.40 ^{NS}	12.20 ^{NS}	1.40 ^{NS}
Treatment	4	402.90 **	27.60 **	51.60**	75.00**
Error	8	10.85	2.65	3.95	5.90

* = Significant at 5% level of probability
** = Significant at 1% level of probability
NS = Non significant