SOMATIC EMBRYOGENESIS OF STEVIA
(*Stevia rebaudiana* Bert.)

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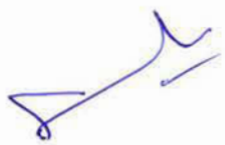

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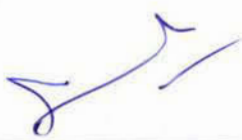

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CERTIFICATE

This is to certify that the thesis entitled, **"SOMATIC EMBRYOGENESIS OF STEVIA (*Stevia rebaudiana* Bert.)** submitted to the Faculty of **AGRICULTURE**, Sher-e-Bangla Agricultural University, Dhaka, in partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE in HORTICULTURE** embodies the result of a piece of bona fide research work carried out by **MD. ATAUR RAHMAN, Registration No. 26191/00485,** under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.

I further certify that any help or source of information, received during the course of this investigation have been duly acknowledged.

Dated: 30.06.07
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Dr. Md. Al-Amin
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Dedicated to
My
Beloved Parents

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ABSTRACT

The experiment was conducted at the Laboratory of Biotechnology Division, Bangladesh Agricultural Research Institute, Gazipur, Bangladesh. Leaf and nodal segments were cultured on MS media supplemented with varying concentrations (0.0, 0.1, 0.2, 0.3 and 0.4 mg/L) of 2,4-D for callus induction. The highest amount of callus 26.38%, 48.50% and 72.15% at 14, 21 and 28 days after inoculation in dark phase was produced in the MS medium with 0.4 mg/L 2,4-D. Interaction effects of media supplemented with NAA (α-napthanlene acetic acid), Kn (Kinetin) and BAP (6-benzyl amino purine) were significant for somatic embryo induction. Among the three media the highest amount of somatic embryo was obtained in MS media supplemented with 5.0 mg/L Kn, 10.0 mg/L Kn and 1.0 mg/L BAP respectively. The highest amount of plant regeneration was obtained in MS media supplemented with 0.1 mg/L NAA + 2.0 mg/L BAP. The best response towards root number and root length was obtained from MS media supplemented with 0.2 mg/L IBA. Finally the regenerated plantlets were successfully established into the pots after proper hardening.

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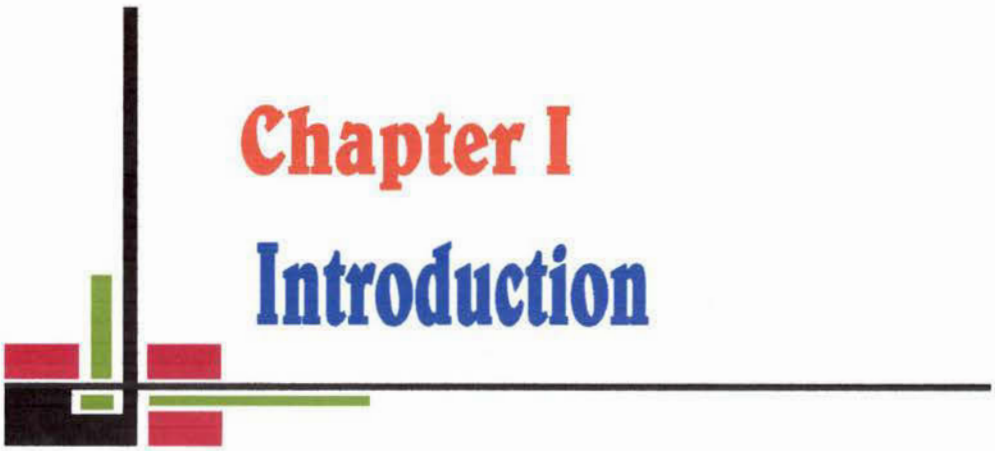
LIST OF ABBREVIATIONS

et al.	*et alia alii* (Latin) = and other people
viz.	*Videlicet* = namely
µM	micro Mole
mM	mili Mole
ppm	parts per million
In vitro	within the test tube
Ex vitro	outside the test tube
MS	Murashige and Skoog
N	Normal solution
g/L	gram(s) per litre
ml	mili litre(s)
mg/L	mili gram(s) per litre
mm	mili metre(s)
cm	centi metre(s)
°C	Degree Celsius
h	hour(s)
2,4-D	2,4-Dichlorophenoxy acetic acid
GA	Gibberellic acid
Kn	Kinetin
IAA	Indole 3-acetic acid
IBA	Indole 3-butyric acid

BA	6-benzyleadenine
BAP	6-benzyl amino purine
NAA	α-napthalene acetic acid
g	gram(s)
Kg	Kilogram(s)
DAT	Days After Transfer
Fig.	Figure
BARI	Bangladesh Agricultural Research Institute



Chapter I

Introduction

CHAPTER 1

INTRODUCTION

Stevia rebaudiana Bertoni (a member of family Asteraceae) is a small perennial herb native to Paraguay and Southern Brazil (Kinghorn and Soejarto, 1985).

The leaves of *Stevia rebaudiana* are 30 times sweeter than sugar, with zero calories. Whereas, pure extract is 300 times sweeter than sugar (Duke, 1993 and Bhosle, 2004). It is also non-caloric, non-fermentable and does not darken upon cooking (Crammer and Ikan, 1986).

Eight types of steviolglycosides are found in leaves *viz.*, rebaudioside A, rebaudioside B, rebaudioside C (dulcoside B), rebaudioside D, rebaudioside E, steviolbioside and dulcoside A. The four major sweeteners are stevioside, rebaudioside A, rebaudioside C and dulcoside A. The sweetness of those similar compounds relative to sucrose are 210, 242, 30 and 30 respectively (Kinghorn, 1992).

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 and Morita, 1987).

Unlike many low-calorie sweeteners, stevioside is stable at high (100 ^{0}C) temperatures and over a range of pH values (pH 3-9) (Kinghorn and Soejarto, 1985).

Stevioside and rebaudioside A were tested for stability in carbonated beverages and found to be both heat and pH stable (Chang and Cook, 1983).

Both stevioside and rebaudioside A are synergistic in mixtures with other high potency sweeteners such as aspertame and good candidates for inclusion in blends (Schiffman *et al.*, 1995).

The sweet compounds pass through the digestive process without chemically breaking down, making Stevia safe for those who needed to control their blood sugar level (Das *et al.*, 1992).

Stevia is an obligate short day plant with a critical day length of about 13 h. Extensive variability within populations for day length sensitivity were reported (Valio and Rocha, 1966 and Zaidan *et al.*, 1980). Stevia is harvested just prior to flowering when steviol glycoside content in the leaves is at its maximum (Sumida, 1968 and Xiang, 1983). Yield of sweetening compounds present in leaf tissue varies according to method of propagation (Tamura *et al.*, 1984b), day-length (Metivier and Viana, 1979) and agronomic practices (Shock, 1982). In subtropical regions of the world stevioside concentration is high in stevia plant, which may be related to cultivation under long days. Concentration of stevioside was similar to that found in samlpes originating in Japan where long days are experienced during the growing season (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 1970s by a Japanese consortium formed for the purpose of commercializing stevioside and stevia extracts (Kinghorn and Soejarto, 1985).

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). Its recent release for human use in Brazil and the possibility of its adoption in other countries as an alternative to common sugar and artificial sweeteners (Brandle and Rosa, 1992) has made stevia cultivation economically viable.

The plant is propagated by seed / cutting or division of mother plants kept during winter in the greenhouse every year. Plant propagation by seed is not very efficient because of low seed fertility (Carneiro *et al.*, 1997) and self incompatibility of the flowers (Miyagawa *et al.*, 1986). Oddone (1997) considers stevia to be self incompatible and insect pollinated. Further, he considers "clear" seeds to be infertile. Poor seed germination is one of the facts limiting large scale cultivation of stevia (Shock, 1982; Duke, 1993 and Carneiro *et al.*, 1997).

Vegetative propagation techniques, such as clump division, multiplication by cutting and micro propagation are in usual practice. The first technique is limiting on account of the low number of new plants that can be obtained simultaneously from a donor plant (Handro and Ferreira, 1989). Propagation by cutting (Tamura *et al.*, 1984a) and micro propagation (Tamura *et al.*, 1984b) showed promising applications since high morphological similarity together with minimal variations in stevioside content were observed in the new plants produced. On account of this, micro propagation technique was selected for use on *Stevia rebaudiana* Bertoni. Plant regeneration from *in vitro* culture can be obtained by embryogenesis or organogenesis. In stevia, regeneration has been obtained by organogenesis from different explants: leaves (Ferreira and Handro, 1988a; Ferreira and Handro, 1987 and Yang and Chang, 1979),

axillary shoots (Bespalhok *et al.*, 1992), stem tips (Tamura *et al.*, 1984b), suspension cultures (Ferreira and Handro, 1988b and Bondarev *et al.*, 2001) and anther (Flachsland *et al.*, 1996). Floret explants also responded in callus induction (Bespalhok and Hattori, 1997). However, somatic embryogenesis has been reported previously from leaves (Bespalhok *et al.*, 1993 and Wada *et al.*, 1981) and stems (Miyagawa *et al.*, 1984).

Therefore the study was undertaken for with the following objectives:

Objectives:

1. Standardization of media for somatic embryogenesis of *Stevia rebaudiana.*
2. *Ex-vitro* establishment of micro-propagated *Stevia rebaudiana.*

Chapter II

Review of literature

CHAPTER 2

REVIEW OF LITERATURE

The world wide demand for high potency sweeteners are increasing. The sweet herb *Stevia rebaudiana* Bertoni produces such sweetener in its leaves with some added advantages. So, it received much attention of the researchers throughout the world to develop its propagation and production technologies, to evaluate sweetening properties and to detect toxicological properties. Some of the available research works related to the present studies have been reviewed in this chapter.

Micropropagation and embryogenesis are two principal pathways of the plant regeneration *in vitro*. Both resulting the production of non chimeric and true to type plants that comprise clonal populations and have been used to develop efficient methods for rapid clonal propagation of a wide variety of herbaceous dicotyledonous and evergreen species. Somatic embryogenesis has been recognized as the efficient method for *in vitro* mass propagation of plants (Parrot *et al.*, 1991; Gray and Purohit, 1991 and Durzan and Durzan, 1991). The main advantages of somatic embryogenesis over axillary or adventitious shoot propagation is possibility of potentially unlimited production of single individuals with functional shoot and root poles.

The effect of nutrient medium components (mineral salts, vitamins, plant growth regulators, sucrose) on the growth of *S. rebaudiana* plants *in vitro* was investigated by Sikach (1998). The composition of the nutrient medium influenced plant growth rate, dry substance content, use of N, P and K from the medium and the contents of diterpene glycosides in plants. The quality (content

of diterpene glycosides) of *S. rebaudiana* can be regulated by changes in the composition of the nutrient medium.

Yurtaeva and Lobov (1998) reviewed that twelve variants of callus culture of *S. rebaudiana* were obtained on substrates containing various amount of growth regulators. Results are presented of analyses of the physiological and cytomorphological characteristics of the 12 variants including changes in fresh and dry weight of callus, changes in the number of cells, mitotic activity and variability.

The composition and content of steviol-glycosides (SGs) in *in vitro* cultures of stevia (*S. rebaudiana*) were investigated by Bondarev *et al.* (2001). A comparative analysis of production of these compounds in intact plants, *in vitro* plants, the differentiated callus and suspension cultures, morphogenic callus and *in vitro* regenerated shoots were conducted. Qualitative composition of the SGs *in vitro* plants was found to be identical to that of intact plants, but their content in the former plants appeared to be about five or six times lower. A significant decrease in this value was not observed upon long term cultivation (for about 5 years) of the plants. Non-differentiated cell cultures, such as callus and cell suspension, were shown to synthesize only minor amounts of the SGs and their content varied greatly during the growth cycle of the culture. Qualitative composition of the SGs in the cell cultures appeared to be highly scant as compared with that of the donor plants. No correlation between the SG content in organs of the donor plants and that in the cell cultures obtained was found. Factors determining plant cultivation conditions and influencing the accumulation of both fresh and dry cell biomass were not able to completely induce the SG synthesis in non-differentiated cell cultures. This process was found to be restored only after the appearance of morphogenic structures and shoot formation.

Bondarev *et al.* (1998) observed that the effects of growth regulators such as NAA, 2,4-D, Kn and BA on the callusogenesis and growth of *S. rebaudiana* (a perennial grass which contains diterpenoid glucosides 300 times as sweet as sucrose) cultured cells. It was observed that on leaf blades the callus formed was 2 to 3 times larger than that on stem fragments. NAA was more effective for cell culture growth than 2,4-D, while BA exhibited a stronger effect than Kn. A significant retardation of callus growth and complete inhibition of organogenesis took place at 2,4-D concentrations ranging from 0.2 to 1.0 mg/L, whereas NAA at similar concentrations markedly stimulated callus growth. The rate of primary callus growth on leaf and stem explants was the highest at NAA to BA ratios of 2.0 mg/L:2.0 mg/L and 4.0 mg/L:1.0 mg/L, respectively. Analogous concentrations of these growth regulators were also found to be effective for further cell subculturing. The combination of NAA and BA was most suitable for the induction of shoot formation by *S. rebaudiana* explants, although the optimal concentration of these compounds was tenfold lower (0.1 - 0.2 mg/L). The addition of gibberellic acid to callus and suspension cultures resulted in a significant increase in their fresh weight due to cell expansion and hydration.

A multiple shoot culture was induced from nodal segments of *S. rebaudiana* on MS (Murashige and Skoog, 1962) medium containing half-strength macroelements, 1% sucrose and supplemented with NAA (0.01 mg/L). A bioreactor with hormone-free MS medium (300 ml) was inoculated with 1.5 g of the multiple shoot culture and cultivated for a month. Culture of the multiple shoot culture in the bioreactor and transfer to *ex vitro* conditions took about 8-9 weeks. A total of 600 plantlets were produced which could be transferred from greenhouse to field conditions (Nepovim and Vanek, 1998).

Stevioside accumulation in callus cultures derived from *S. rebaudiana* leaf explants was positively correlated with cell organization and greening of the cultures but negatively correlated with callus growth rate reported by Xie *et al.* (1998). Callus shoot formation was not essential for stevioside synthesis. Stevioside content was highest in slow-growing, compact, green calluses with or without shoot formation (5.78% and 5.37%, respectively). Cells of these calluses were highly vacuolated and contained fully developed chloroplasts with dense stromata and plastoglobuli. Microbodies containing crystal lattices were closely associated with chloroplasts. Plastids in yellow, compact callus cells, which accumulated less stevioside (2.13%) than green calluses, contained many starch grains with few dispersed lamellae. Stevioside content was lowest (0.96%) in fast-growing, loose, yellow calluses, in which cell plastids were structurally simple and contained few lamellae. It is concluded that stevioside accumulation is related to plastid development and vacuole differentiation.

Bespalhok and Hattori (1997) studied Somatic embryos were obtained from floret explants of *S. rebaudiana* cultured on Murashige and Skoog medium supplemented with 2,4-D (9.05 or 18.10 μM) and Kn (0-9.29 μM). On 9.05 μM 2,4-D supplemented medium without Kn, maximum embryogenic callus formation occurred. On 18.10 μM 2,4-D supplemented medium, the best results were obtained with 2.32 μM Kn. Callus formation started at the base of the corolla and ovaries. Histological sections showed a fibrillar network on the surface of somatic proembryos. An unicellular origin for the somatic embryos is proposed.

In vitro nodal segments of 6-week-old seedlings were cultured on MS medium with 50% macroelement content and in the presence of 0.1 ppm NAA. 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 (Acuna *et al.*, 1997).

Kornilova and Kalashnikova (1996) conducted an experiment and found that a laboratory procedure was developed for the clonal micropropagation of *S. rebaudiana*, using stem segments with two axillary buds. Use of MS medium without growth regulators gave results comparable to those obtained with growth regulators (BA + NAA and Kn + NAA). Accordingly, plain MS medium is recommended as being cheap and effective. IAA at 0.5 mg/L gave good results in activating rhizogenesis. No reduction in growth was observed with increasing number of subcultures *in vitro*.

Callus tissue cultured in liquid medium in the light contained chloroplasts reported by Xie *et al.* (1995a). Transfer onto fresh medium resulted in the decrease or disappearance of photosynthetic lamellae and the eventual dedifferentiation of chloroplasts to form proplastid-like structures. This was correlated with the degradation of internal membranes and the dilution of stroma constituents and thylakoid membranes due to chloroplast divisions. The process was not synchronous so that some plastids retained intact lamellae when others had lost them completely. Also some plastids contained starch grains. A striking feature of this process was the apparent grouping of organelles around the nucleus. After seven days of continuous culture, many cells appeared to be meristematic, with cytoplasm rich in organelles filling the cell, and most of the plastids found were proplastids with an internal structure consisting of randomly positioned single thylakoids. During the stationary phase of cell growth, the internal structures became organized into stacked

grana and the plastid ribosomes increased. The correlation between dedifferentiation of the highly vacuolar cells induced by the subculture process and the dedifferentiation of the chloroplasts is discussed.

Xie *et al.* (1995b) studied transfer of callus cultures to a differentiating medium containing BA caused marked changes. Cells within the meristematic regions formed in the callus were small and had large nuclei, some with intranuclear inclusions, with both prominent and vacuolated nucleoli. Numerous small vacuoles were distributed around the cell periphery or dispersed through the cytoplasm. Dictyosomes were common and sometimes appeared in groups. Multiple and double membrane-bound concentric structures derived from the endoplasmic reticulum were often seen. The cytoplasm contained ribosomes sometimes seen as clusters of polysomes. Another distinctive feature was the appearance of plasmalemma invaginations. It was concluded that these ultrastructural changes reflected morphological changes which preceded organ differentiation in callus culture.

Somatic embryos were induced when leaves were cultured *in vitro* on MS medium supplemented with 2,4-D (10 or 25 μM) and BA (1 μM) and a high sucrose concentration (120 g/L) studied by Bespalhok *et al.* (1993) . The embryos appeared to be formed directly without intermediate callus development. Somatic embryos failed to mature and developed roots but not shoots when transferred to MS medium without growth regulators and with a low (30 g/L) concentration of sucrose.

Leaf explants of *S. rebaudiana*, cut into 1 cm^2 sections after surface sterilization, were cultured on MS medium containing different growth substances, at 25°C and 2000 lux, with a 12 h photoperiod. Results showed that stevioside content in differentiated callus was higher than that in

undifferentiated callus. The ability to synthesize stevioside in callus derived from the dedifferentiation of leaves decreased markedly. The stevioside content of leaves of plants derived from the differentiation of callus was twice than that of plants cultured in the field. The content of stevioside was highest in callus cultured on medium supplemented with 1 ppm GA; supplementation with IAA or NAA was less effective (Chen and Li, 1993).

Bespalhok *et al.* (1992) revealed that two cm long nodal segments were excised from adult *S. rebaudiana* plants and cultured for shoot proliferation on MS medium containing six levels of NH_4NO_3, the Fossard vitamins, 11 µM BA, 3% sucrose and 1% agar. When shoots were about 5 cm long they were transferred for rooting to full- or half-strength MS medium containing NAA at 0.0 to 10.0 µM. Lowering the NH_4NO_3 concentration in the multiplication medium from the standard 20.60 mM to 5.15 mM increased the number of shoots produced per nodal segment to an average of 10.90. The standard MS concentration of NH_4NO_3 also induced toxicity symptoms. Decreasing the MS salt level in the rooting medium by half increased the number of roots/shoot and at this level 1.0 and, particularly, 10 µM NAA had a beneficial effect on root induction. The survival rate of rooted plants on transfer to potting medium was 95%.

Isolated shoot primordia of *S. rebaudiana* were used as the inoculum to obtain clusters of shoot primordial studied by Akita *et al.* (1994). Such clusters were cultured in a 500 litre bioreactor to obtain shoots. A total of 64.6 kg of shoots were propagated from 460 g of the inoculated shoot primordia. These shoots were easily acclimatized in soil.

Srivram and Mukandan (2003) regenerates shoots from shoot apex, nodal and leaf explants of *S. rebaudiana* Bertoni by culturing them on Murashige and

Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 6-benzyleadenine (BA) 8.87 at μM and indole-3-acetic acid (IAA) 5.71 at μM. Rooting of the *in vitro* derived shoots could be achieved following subculture onto auxin containing medium. A survival rate of 70% was recorded at the hardening phase on the substrate cocopeat. The presence of the sweet diterpene glycosides, *viz.* stevioside and rebaudioside, was confirmed in the *in vitro* derived tissues of stevia using HPTLC techniques. Callus cultured on agar-solidified MS medium supplemented with BA (8.87 μM) and indole-3-butyric acid (9.80 μM) showed the highest sweetener content.

Sritongkum (1995) developed the procedures for micropropagation from primary leaf and shoot tip of *S. rebaudiana* Bertoni. The optimal conditions for callus induction from primary leaf were observed on MS medium supplemented with 2.0 mg/L NAA and 2.0 mg/L IBA. The optimal conditions for primary leaf callus regeneration was achieved on MS medium containing Nitsch (Nitsch and Nitsch, 1969) vitamins supplemented with 2.0 mg/L NAA and 2.0 mg/L BA. Root formation was occurred on MS medium supplemented with 0.1 mg/L NAA. The efficiency of adventitious shoot formation could be increased to a number of 60 shoots per callus within three months. Shoot multiplication from shoot tips was observed on MS medium supplemented with 12.0 mg/L Kn. The optimal condition for root induction was occurred both in MS medium supplemented with 0.01 mg/L NAA and MS medium without plant regulator. Plantlets could be transplanted to potting soil.

Salas (2001) found that the most effective explants for large scale production of the plants appeared to be micro-cutting with apical or axial buds. They were successfully cultivated on the hormone-free medium and this allowed producing the plants by several thousands for 3-4 months.

Protocols for regeneration of *S. rebaudiana* via somatic embryogenesis was developed by Pute (1992). It is important as this technique can be used in the clonal propagation of this plant or as explant material for protoplast isolation and regeneration.

In vitro propagation of stevia was attempted in two methods were studied by Kabir (2005). The first method was 'Direct *in vitro* propagation' by multiple shoot regeneration from the explants (shoot tip and nodal segments) followed by rooting of the multiplied shoot where the second method was 'Indirect *in vitro* propagation' by callus induction on the explants (leaf disk, nodal segment and internodal segment) followed by shooting and rooting of the resulted callus. In 'Direct *in vitro* propagation' MS media in two different strengths (full MS and ½ MS) were used as culture media for shoot proliferation as well as for root induction. Cytokinins *viz.* BAP and Kn were used for shoot proliferation in four concentrations (0.0, 1.0, 2.0 and 3.0 mg/L). Auxins *viz.* NAA and IBA were used for root induction for microcuttings in four concentrations (0.0, 0.5, 1.0 and 1.5 mg/L). In 'Indirect *in vitro* propagation' MS media supplemented with 2,4-D, NAA and BAP at four concentrations (1.0, 3.0, 5.0 and 7.0 mg/L) were used for callus induction and then subsequently subcultured onto MS media containing 1.0 mg/L BAP and 1.5 mg/L NAA for shoot and root induction of the callus. Shoot tip explants showed better response for shoot proliferation than nodal segment. MS media in full and half strength were equally effective shoot proliferation. BAP (1.0 mg/L) was superior to all other hormonal treatments for shoot induction. For root induction, MS full strength was superior to half strength. MS full strength supplemented with NAA (1.5 mg/L) was the best medium for rooting of micro-cuttings. In callus induction, leaf disk explant was superior to inter node and nodal segment. MS media supplemented with BAP (7.0 mg/L) was the best medium for the callus formation.

Huda (2006) conducted an experiment that leaf segments, inter node and nodal segments of *S. rebaudiana* were cultured on MS media supplemented with various concentrations (1.0, 3.0 5.0 and 7.0 mg/L) of growth regulators (2,4-D, BAP and NAA) along with coconut water for morphogenic responses mainly through callus culture. Ninety five per cent aseptic cultures were obtained when sterilized with 0.1% $HgCl_2$ for 10 minutes. Nodal segment initiated callus earlier than leaf segments and inter node exlant. Higher amount of callus were obtained from leaf segments than inter node and nodal segment. Explant of *S. rebaudiana* supplemented with coconut water and 2,4-D mainly facilitated callus formation but in case of BAP, shoot along with root formation occurred and incase of NAA root along with callus formation occurred. Interaction effects of explant and growth regulators were significant for days of callus initiation, fresh weight and dry weight of callus per culture. Among the twelve treatments the highest amount of callus was obtained in MS media supplemented with 7.0 mg/L BAP.

Islam (2004) reviewed shoot tip, nodal segment and leaf base with petiole of *S. rebaudiana* were cultured on MS media supplemented with various concentrations of BA for shoot production. Nodal segment initiated shoots earlier than shoot tip explant. Higher number of shoot per culture and micro-cutttings were obtained from shoot tip explant than nodal segment. Leaf base with petiole failed to produce shoot on any of the media used. The highest number of shoots (3.2) and micro-cuttings (5.8) were found in the MS medium with 3.0 mg/L BA and 2.0 mg/L BA respectively. Interaction effect of explant and shooting media were significant for days of shoot initiation, number of shoot per culture and number of micro-cuttings per culture. Among the four media the highest number of root per shoot was obtained in MS medium supplemented with 1.0 mg/L NAA followed by 1.5 and 0.5 mg/L NAA respectively. No roots were produced in the medium without auxin (NAA).

Chapter III

Materials and Methods

Micropropagation method for *Stevia rebaudiana* plants was established by culturing nodal segments on Murashige and Skoog (MS) medium supplemented with 6-benzyl amino purine (BAP) or kinetin (Kn) at different concentrations were investigated by Tadhani *et al.* (2006). Initiation of shoot induction was observed within 4-5 days. The maximum number of shoots was obtained on MS medium supplemented with 0.6 mg/L BAP. Optimum concentration of Kn was 4.0 mg/L for maximum shoot production. The obtained shoot cultures were transferred for rooting on MS medium supplemented with various concentrations of Indole 3-butyric acid (IBA). Initiation of rooting was observed within 6-7 days. Maximum number of roots was observed on the medium supplemented with 1.0 mg/L IBA. The regeneration of plantlets was successfully hardened in soil: sand (1:1).

CHAPTER 3

MATERIALS AND METHODS

The experiment was conducted at the Laboratory of Biotechnology Division, Bangladesh Agricultural Research Institute, Gazipur, Bangladesh during the period of September, 2006 to March, 2007. The materials and methods used in these investigations are described below:

3.1 Plant materials:

Leaf and nodal segments were collected from Biotechnology Division, Bangladesh Agricultural Research Institute, Gazipur, Bangladesh and maintained under laboratory conditions. Leaf side and nodal segments from plants were cut and rinsed in running water. The explants were surface sterilized in laminar air flow cabinet, treated with 70% alcohol for 2 minutes followed by rinsing with sterile distilled water which were followed by treatment with 0.1% mercuric chloride for 2 minutes. Finally the explants were washed with sterile distilled water successively three times and inoculated under aseptic conditions.

3.2 Preparation of culture medium:

The first step in the preparation of the media is the preparation of stock solutions. A nutrient medium for somatic embryogenesis usually consists of inorganic and organic salts, iron, a carbon source, vitamins and growth regulators (Table 1).

3.2.1 Preparation of stock solutions:

Stock solutions of all components were prepared with appropriate amount of all the components. Appropriate amount of all the stock solutions were mixed with the particular prescribed amount of medium following the standard media preparation procedure.

Table 1. Constituent of stock solutions for MS (Murashige and Skoog, 1962) medium

Constituents	Concentrations (mg/L)
Macronutrients (10x)	
KNO_3	1900.0
NH_4NO_3	1650.0
KH_2PO_4	170.0
$CaCl_2.2H_2O$	440.0
$MgSO_4.7H_2O$	370.0
Micronutrients (100x)	
$MnSO_4.4H_2O$	22.30
H_3BO_3	6.20
$ZnSO_4.7H_2O$	8.60
KI	0.83
$Na_2MoO_4.2H_2O$	0.25
$CuSo_4.5H_2O$	0.25
$CoCl_2.6H_2O$	0.025
Iron sources (10x)	
$FeSO_4.7H_2O$	27.80
Na_2-EDTA	37.30
Organic nutrients (100x)	
Glycine	2.00
Nicotinic acid	0.50
Pyridoxine-HCl	0.50
Thaimine-HCl	0.10
Myo-inositol	100.00

To prepare the different stock solutions the same procedure was followed everywhere. Preparation of the different stock solutions is briefly described below:

3.2.1.1 Stock solution A (Macronutrients):

Macronutrients solution was made up to 10 times (10x) of the final strength of the medium in 1000 ml of distilled water. The amount of salts required per litre of the medium was weighed accurately and dissolved in 750 ml of distilled water and final volume was made up to 1000 ml by further addition of distilled water. This stock solution was filtered and poured into a clean reagent bottle and stored in a refrigerator at 4°C for later use.

3.2.1.2 Stock solution B (Micronutrients):

The stock solution of micronutrients was made up to 100 times (100x) of the final strength of the medium in 1000 ml of distilled water. The stock solution was filtered, labeled and stored in a refrigerator at 4°C for later use.

3.2.1.3 Stock solution C (Iron source):

The stock solution was prepared at100 times (100x) of the final strength of $FeSO_4$ and Na_2-EDTA in 100 ml of distilled water and dissolved by heating on a heater cum magnetic stirrer. Then the volume was made up to 1000 ml by further addition of distilled water. Finally, the stock solution was filtered and stored in a refrigerator at 4°C for experimental use.

3.2.1.4 Stock solution D (Vitamins):

Each of the desired ingredients except myo-inositol were taken at 100 times (100x) of their final strength in a measuring cylinder and dissolved in 400 ml of distilled water. Then the final volume was made up to 1000 ml by further addition of distilled water. The solution was dispensed into 10 ml aliquots and stored at 4°C. The desired concentration of myo-inositol was added directly into the culture medium during its preparation.

3.2.1.5 Stock solution of hormones:

Stock solutions of different hormones were prepared by dissolving the specific hormone of desired quantity to the appropriate solvent and made the final volume with distilled water. The following hormonal supplements were used in the present investigation.

Auxins:

Indol-3 butyric acid (IBA)

α-napthalene acetic acid (NAA)

2,4-Dichlorophenoxy acetic acid (2,4-D)

Cytokinins:

6-benzyl amino purine (BAP)

Kinetin (Kn)

These hormonal supplements were dissolved in proper solvents against each of them as shown below:

Hormones (Solute)	Solvents
IBA	70% ethyl alcohol
NAA	0.1 N NaOH
2,4-D	70% ethyl alcohol
BAP	0.5 N HCl
Kn	0.5 N HCl

To prepare a stock solution of any of these hormones, 10.0 mg of hormone was taken on a clean watch glass and then dissolved in 1.0 ml of particular solvent and collected in a 100 ml measuring cylinder and the volume was made up to 100 ml with distilled water. The solution was then poured into a clean plastic container and stored at 4°C and used for a maximum period of two weeks.

After the preparation of stock solution, the later step was the preparation of culture media. To prepare 1.0 litre of MS medium, the following steps were carefully maintained.

i) At first a conical flask was taken and 100 ml of macronutrients, 10 ml of micronutrients, 10 ml of iron and 10 ml of vitamins were accurately pipetted into this flask and stirred on a heater cum magnetic stirrer to mix these ingredients properly.

ii) 500 ml of distilled water was added in the flask to dissolve all the ingredients.

iii) 100 mg of myo-inositol was added directly to the solution and dissolved well (only for MS medium).

iv) 30 g of sucrose (i.e. 3% of sucrose) was added to this solution and gently agitated to dissolve completely.

v) Based on the culture medium, different concentrations of hormonal supplements were added to the solution either in single or in combination as required and mixed well. Since 100 ml of each hormonal stock solution contained 10 mg of hormonal salts, the addition of 10 ml of any hormonal stock solution to prepare 1.0 litre of medium resulted in 1.0 mg/L concentration of these hormonal supplements.

vi) The solution was then poured into a 1.0 litre measuring cylinder and the volume was made up to 1000 ml with addition of distilled water and poured back to a 2.0 litre conical flask.

Vii) The pH of the medium was adjusted to 5.8 with the help of 0.1 N NaOH or 0.1 N HCl, whichever was necessary.

Viii) About 0.6% (i.e. 6 g) of Bacto agar (Difco-brand) was added to solidify the medium. The mixture was then gently heated with continuous stirring until complete dissolution of agar.

Ix) Required volume of hot medium was then dispensed into culture vessels *viz.* test tubes or baby jars. After dispensing the medium the test tubes or

the baby jars were capped as early as possible with aluminium foil or poly propylene sheets and marked with different codes with the help of marker to indicate the specific medium.

3.3 Culture media:

3.3.1 Callus induction and explants culture medium:

MS (Murashige and Skoog, 1962) media supplemented with various concentrations of 2,4-D (0.0, 0.1, 0.2, 0.3 and 0.4 mg/L) were prepared for the induction of embryogenic callus from *S. rebaudiana* explants. After mixing all stock solutions 3% sugar was added for the MS media preparation. Then the pH of the media was adjusted to 5.8. The 0.6% agar was dissolved and the media was heated and dispensed in the test tube and capped with aluminium foil. Test tube containing media were autoclaved at 121°C at 1.1 Kg/cm^2 for 20 minutes.

3.3.2 Somatic embryogenesis medium:

MS (Murashige and Skoog, 1962) media supplemented with various concentrations of BAP (0.0, 1.0, 2.0 and 3.0 mg/L), Kn (0.0, 5.0, 10.0 and 15.0 mg/L) and NAA (0.0, 0.1, 0.2 and 0.3 mg/L) were prepared for the induction of somatic embryo from embryogenic callus. The 0.6% agar was dissolved, the media was heated and dispensed in the test tube and capped with aluminium foil. Test tube containing mediua were autoclaved at 121°C at 1.1 Kg/cm^2 for 20 minutes.

3.3.3 Plantlet regeneration medium:

MS (Murashige and Skoog, 1962) media supplemented with various concentrations of NAA and BAP were prepared for multiple shoots from somatic embryo. Four levels of NAA (0.0, 1.0, 2.0 and 3.0 mg/L) and 4 levels

of BAP (0.0, 0.1, 0.2 and 0.3 mg/L) were combiendly used to prepare the plantlet regeneration medium. The 0.6% agar was dissolved and the media was heated and dispensed in the test tube and capped with aluminium foil. Test tube containing media were autoclaved at 121°C at 1.1 Kg/cm^2 for 20 minutes.

3.3.4 Root growth medium:

MS (Murashige and Skoog, 1962) media supplemented with various concentrations of IBA (0.0, 0.1, 0.2, 0.3 and 0.4 mg/L) were prepared for the induction of root from multiple shoots. After mixing all stock solutions 3% sugar was added for the MS media preparation. Then the pH of the media was adjusted as above. The 0.6% agar was dissolved and the media was heated and dispensed in the test tube as above and capped with aluminium foil. Test tube containing media were autoclaved at 121°C at 1.1 Kg/cm^2 for 20 minutes.

3.4 Inoculation technique:

General aseptic techniques concerning *in vitro* culture of the explants were followed in the present experiment. After surface sterilization, the explants were cut into 1.0 cm and inoculated callus induction medium (Fig. 1 and Fig. 2). When the callus was fully grown (for about 1 month), the callus was taken out from the test tube in a laminar air flow cabinet and were cut about 1.0 cm. Then the callus was inoculated on somatic embryogenesis medium. When the somatic embryo was fully grown (for about 1 month), the somatic embryo was taken out from the test tube in a laminar air flow cabinet and was cut about 1.0 cm. Then the somatic embryo was inoculated on plantlet regeneration medium. When the shoot was completely grown (for about 1 month) was taken out from the test tube in a laminar air flow cabinet and were cut about 1.0 cm. Then the shoots were inoculated on root growth medium. All inoculations and aseptic manipulations were carried out in a laminar air flow cabinet. Before use, the working surface of the cabinet was cleaned by swabbing with 90% ethyl

alcohol and UV light (for 20 minutes) to reduce the chances of contamination. The instruments like scalpels, forceps, needles, etc. were sterilized by an alcoholic dip followed by flaming inside the laminar air flow cabinet. Other requirements like petri dish, bottles, conical flasks, cotton, distilled water etc. were also sterilized. Before the onset of inoculation, hands were washed thoroughly by soap and then swabbing with 70% ethyl alcohol. Cutting and transfer of the explants were carried out taking all possible care to ensure contamination free inoculation.

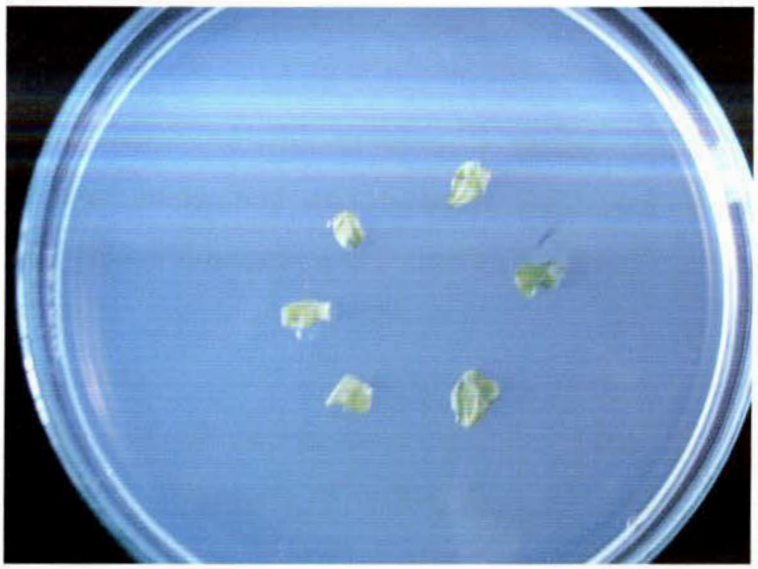


Fig. 1. Initial leaf explants on callus induction medium

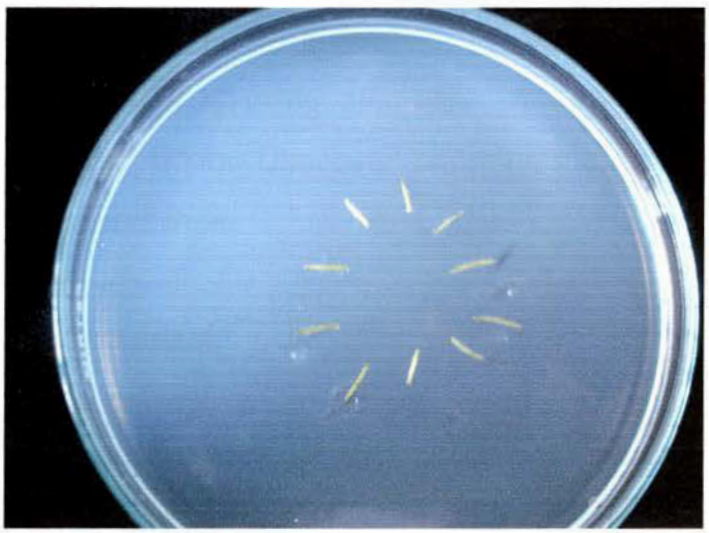


Fig. 2. Initial internode explants on callus induction medium

3.5 Culture environment:

All cultures were grown in an air-conditioned culture room illuminated by 40 W white fluorescent tubes with an intensity varied from 2000-3000 lux. The photoperiod was maintained as 16 hours light and 6 hours dark. The temperature of the culture room was maintained at 25±2°C.

3.6 Data collection:

Visual observation of culture was made every day. Data were recorded for days of callus induction, % growth of callus, days of somatic embryogenesis, % growth of somatic embryo, days of plantlet regeneration, number and length of root per plant. Except number of root per plant and length of root per plant, all data were recorded 14 days, 21 days and 28 days after culture. The data for root number and root length were recorded after one month.

3.7 Data analysis:

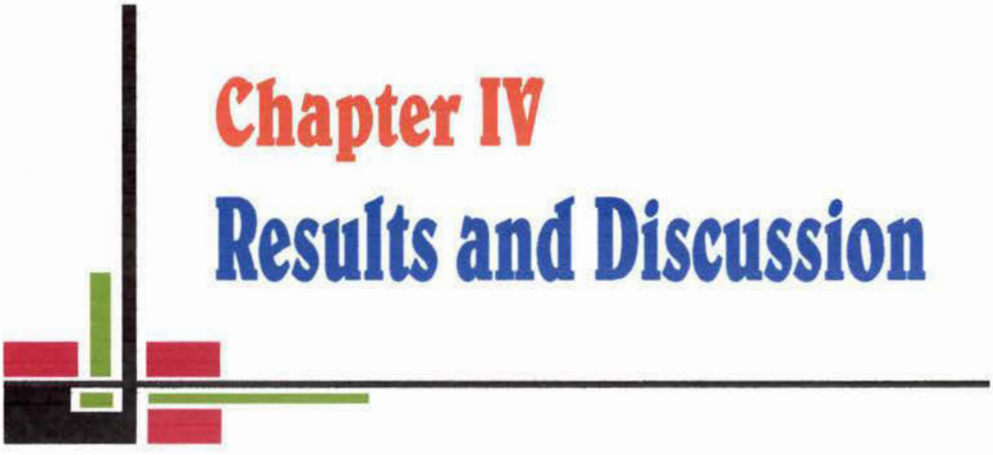
All data collected were assessed by analysis of variance for factorial complete randomized design (CRD) using computer software MSTAT-C (MSTAT Development Team, 1988). Duncan's Multiple Range Test (DMRT) was applied for means separation.

3.8 Hardening of plantlets:

For hardening the *in vitro* generated plantlets were removed carefully and dipped in 0.1% Bavistin (antifungal) for few minutes. Finally the plantlets were transferred on previously autoclaved sand:soil (1:1) mixture. Polyethylene bags were sprinkled with water on the inner layer was used to cover the plant in order to maintain the humidity and the plants were also irrigated with a diluted mixture of basal salts. After 10 days, polyethylene bags were removed and plantlets were hardened for 2-3 weeks in hardening shade.

3.9 Plantation of hardened plantlets:

After 2-3 weeks of hardening of plantlets they become 7-12 inches in height and strong enough for plantation to soil under normal condition. Therefore, they were then transferred to fields or big pots where they were grown. Care was taken of that roots did not get any damage during plantation.



Chapter IV

Results and Discussion

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Callus induction:

Formation of embryogenic callus was first observed at 14 days after inoculation (Table 2). The embryogenic callus was characterized by a light green or light yellow colour, compact structure and presence of globular somatic embryos on its surface (Fig. 3). Non-embryogenic callus was also present characterized by white colour and hyperhydric appearance. Table 2 shows the percentage of explants with embryogenic callus at different 2,4-D concentrations. At 0.0 mg/L 2,4-D, embryogenic callus formation was limited to all explants. On 0.4 mg/L 2,4-D, maximum embryogenic callus (26.38%) was produced at 14 days after inoculation in dark phase and the amount of callus was increased day by day. The maximum embryogenic callus 48.50% and 72.15% was found at 21 and 28 days after inoculation respectively in dark phase (Fig. 4). On the other hand, on 0.4 mg/L 2,4-D, maximum embryogenic callus 23.11%, 45.13% and 59.90% was produced at 14, 21 and 28 days respectively after inoculation in light phase. For floret explants 2,4-D (at 9.05 or 18.10 μM) was necessary for somatic embryogenesis and high levels (4.65-9.29 μM) of kinetin reduced the percentage of explants with somatic embryogenic callus (Bespalhok *et al.*, 1997). Bespalhok *et al.*, (1993) also used leaf explants and 2,4-D for callus induction.

Table 2. Effect of 2,4-D on percentage of callus formed on *in vitro* explants of *Stevia rebaudiana*

Swelling of explants (%)						
2,4-D level (mg/L)	**Dark phase**			**Light phase**		
	14DAT	**21DAT**	**28DAT**	**14DAT**	**21DAT**	**28DAT**
0.0	2.98d	6.45d	9.42d	1.63c	6.39c	8.22b
0.1	24.83a	39.48b	59.92b	20.83b	38.67b	59.67a
0.2	22.15b	35.40c	54.65c	19.92b	38.17b	58.86a
0.3	19.96c	35.38c	52.06c	19.58b	37.00b	57.71a
0.4	26.38a	48.50a	72.15a	23.11a	45.13a	59.90a

In a column, means followed by a common letter(s) under the same factor are not significantly different at 1% level of significance by DMRT.

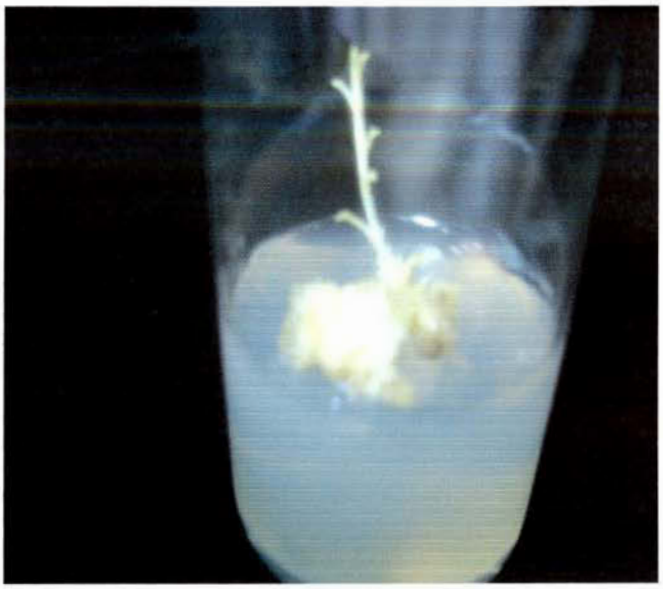


Fig. 3. Light yellow, compact globular structures indicating the initial stage of somatic embryo induction

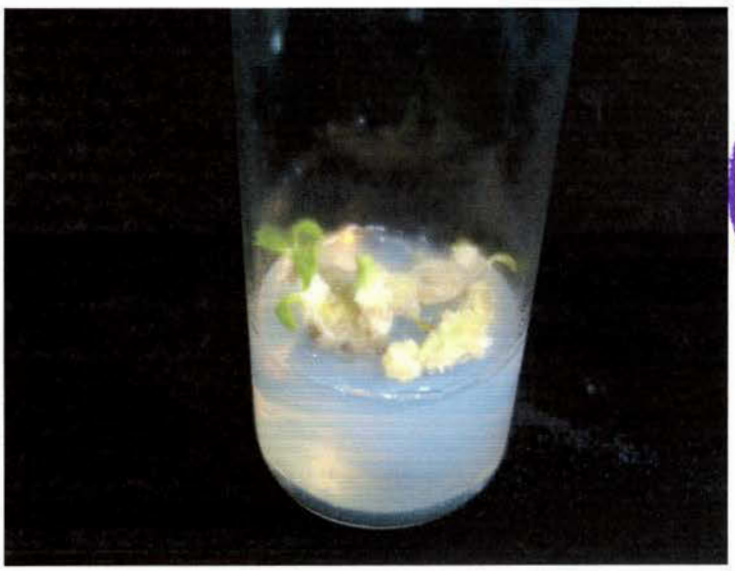


Fig.4. Influence of 2,4-D on explants for callus induction

4.2 Somatic embryo induction:

Somatic embryos were first observed 14 days after callus transfer (Table 3). Somatic embryo production from *in vitro* grown callus was significantly influenced by media used (Fig. 5). Among Kn, BAP and NAA the majority of the somatic embryos were produced in 5.0 mg/L Kn 30.75%, 38.59% and 51.00% for 14, 21 and 28 days after transfer in media respectively. The lowest amounts of somatic embryos were observed in control treatment.

For Kn, 5.0 mg/L Kn was the best concentration for somatic embryo induction, then 15.0 mg/L Kn was the better concentration than the others. Control treatment of Kn was the porest concentration. For BAP, 1.0 mg/L BAP was the best concentration for somatic embryo induction, 18.59%, 37.92% and 40.63% somatic embryos were produced at 14, 21 and 28 days after transfer of callus respectively then 2.0 mg/L BAP was the better concentration than the others. On the other hand 0.2 mg/L NAA was the best concentration for somatic embryo induction, 15.54%, 31.25% and 36.04% somatic embryos were produced at 14, 21 and 28 days after transfer of callus respectively. Then 0.3 mg/L NAA is the better concentration than the others. In case of without NAA, lowest embryos were observed.

Except 0.0 mg/L NAA all the concentrations of NAA (0.1, 0.2 and 0.3 mg/L) had produced somatic embryo with long roots (Fig. 6).

Table 3. Effect of Kn, BAP and NAA callus and percentage of somatic embryos formed in *Stevia rebaudiana*

		Swelling of callus (%)		
		14DAT	21DAT	28DAT
	0.0 mg/L	0.65f	2.29f	0.53f
NAA	0.1 mg/L	16.67d	21.67e	19.38g
	0.2 mg/L	15.54de	31.25b	36.04c
	0.3 mg/L	14.09e	23.00de	32.38d
Kn	5.0 mg/L	30.75a	38.59a	51.00 a
	10.0 mg/L	26.79b	10.96e	21.42d
	15.0 mg/L	25.50b	28.13c	29.88d
BAP	1.0 mg/L	18.59c	37.92a	40.63b
	2.0 mg/L	15.08de	30.33bc	36.09c
	3.0 mg/L	14.33e	25.00d	26.92e

In a column, means followed by a common letter(s) under the same factor are not significantly different at 1% level of significance by DMRT.

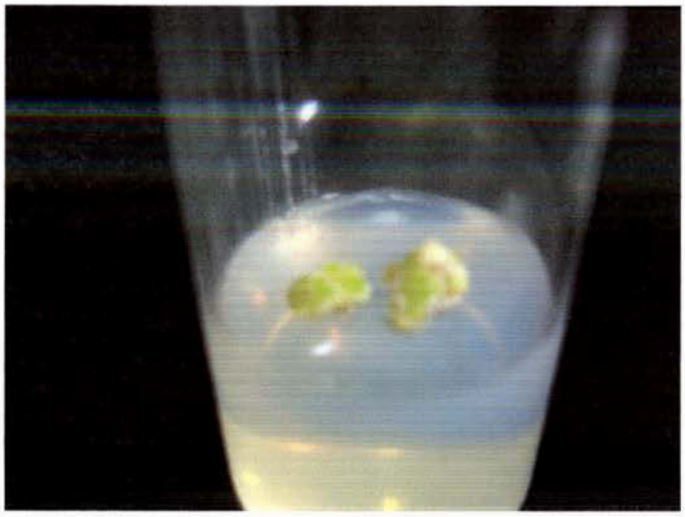


Fig. 5. Influence of Kn on callus for somatic embryo induction

Fig. 6. Embryos with long roots produced by NAA

4.3 Plant regeneration:

Plant regeneration from *in vitro* somatic embryo was significantly influenced by media used. For the plant regeneration NAA and BAP were combinedly used in MS media (Table 4). Among all the concentrations 0.1 mg/L NAA + 2.0 mg/L BAP was the best for plant regeneration. In control treatment, few plant regeneration was observed. In case of 0.3 mg/L NAA + 3.0 mg/L BAP, the maximum amount of plant regeneration (16.88%) was produced at 14 days after transfer of somatic embryo and in case of 0.1 mg/L NAA + 2.0 mg/L BAP the maximum amount of plant regeneration (30.42% and 39.59%) was produced at 21 and 28 days after transfer of somatic embryo respectively (Fig. 7).

Table 4. Effect of NAA and BAP on the somatic embryos and percentage of plant regeneration on *in vitro* somatic embryos of *Stevia rebaudiana*

Treatment NAA + BAP (mg/L)	14DAT (%)	21DAT (%)	28DAT (%)
0.0 + 0.0	3.33f	5.63i	1.04o
0.0 + 1.0	9.37e	14.95g	11.75l
0.0 + 2.0	11.68d	17.92e	25.20h
0.0 + 3.0	15.0bc	25.00b	30.21de
0.1 + 0.0	1.79g	5.04i	7.33m
0.1 + 1.0	12.50d	20.00d	37.63b
0.1 + 2.0	13.96c	30.42a	39.59a
0.1 + 3.0	15.49b	22.08c	31.25cd
0.2 + 0.0	3.33f	8.33h	15.63k
0.2 + 1.0	10.42e	15.0g	29.31ef
0.2 + 2.0	14.38c	20.63cd	27.88fg
0.2 + 3.0	11.88d	17.08ef	22.06i
0.3 + 0.0	9.82e	15.63fg	5.08n
0.3 + 1.0	9.33e	15.0g	17.65j
0.3 + 2.0	14.38c	21.04cd	27.08g
0.3 + 3.0	16.88a	22.21c	32.08c

In a column, means followed by a common letter under the same factor are not significantly different at 1% level of significance by DMRT.

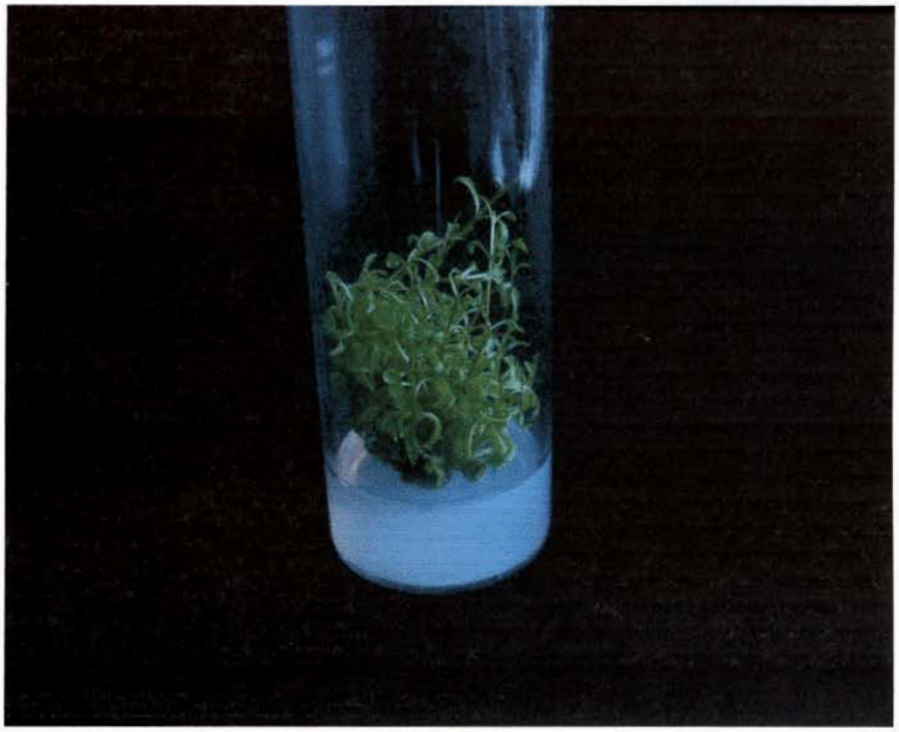


Fig. 7. Germinated embryo produced multiple shoot in NAA and BAP combination media

4.4 Root induction:

Root number and root length produced from *in vitro* grown shoots was significantly influenced by media used (Table 5). Among the different concentrations of IBA tested, 0.2 mg/L IBA was the best for root number and 0.1 mg/L IBA was statistically identical. The same concentration 0.2 mg/L IBA was also the best concentration for root length. Closely followed by 0.1 mg/L IBA (Fig. 8).

Table 5. Effect of IBA on root number and root length of regenerated Plantlets of *Stevia rebaudiana*

IBA (mg/L)	Root number	Root length (mm)
0.0	3.75b	7.83b
0.1	6.79a	8.83ab
0.2	6.81a	9.99a
0.3	4.78b	8.06b
0.4	4.75b	7.90b

In a column, means followed by a common letter under the same factor are not significantly different at 1% level of significance by DMRT.

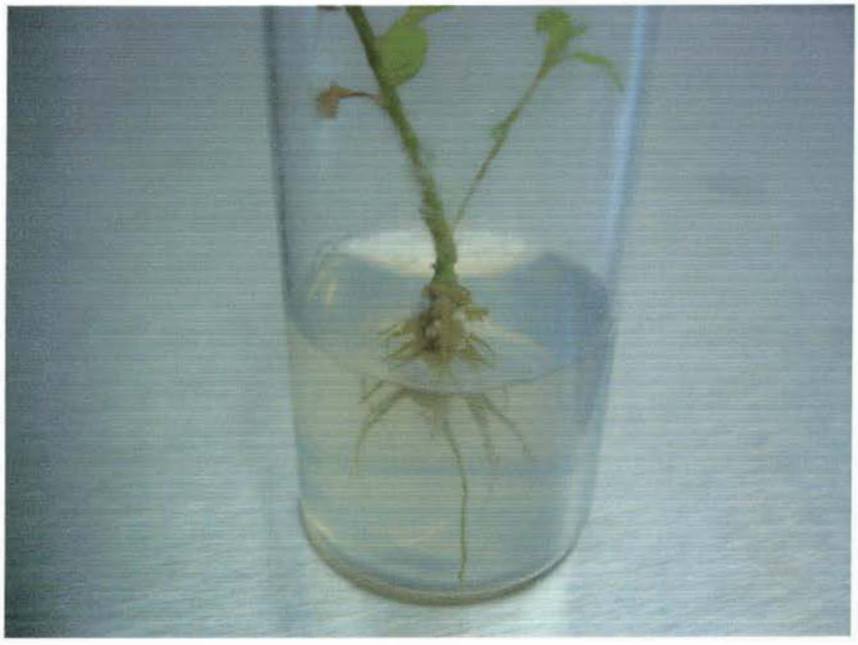


Fig. 8. Influence of IBA on root induction

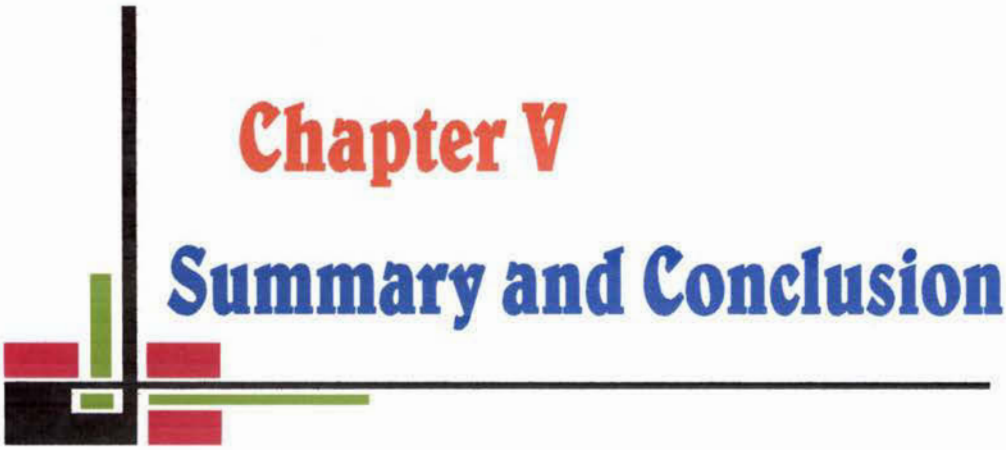
4.5 Hardening of plantlets:

For hardening, the *in vitro* generated plantlets were removed carefully and dipped in 0.1% Bavistin (antifungal) for few minutes. Finally the plantlets were transferred on previously autoclaved sand:soil (1:1) mixture. Polyethylene bags were sprinkled with water on the inner layer was used to cover the plant in order to maintain the humidity and the plants were also irrigated with a diluted mixture of basal salts of stock solution A. After 10 days, polyethylene bags were removed and plantlets were hardened for 2-3 weeks in hardening shade.

4.6 Plantation of hardened plantlets:

After 2-3 weeks of hardening of plantlets they become 7-12 inches in height and strong enough for plantation to soil under normal condition (Fig. 9). Therefore, they were then transferred to fields or to big pots where they were grown. Care was taken of that roots did not get any damage during plantation.

Fig. 9. Established plantlet of stevia



Chapter V

Summary and Conclusion

CHAPTER 5

SUMMARY AND CONCLUSION

The experiment was conducted at the Laboratory of Biotechnology Division, Bangladesh Agricultural Research Institute, Gazipur, Bangladesh from September 2006 to March 2007. Leaf and nodal segments were used for *in vitro* plant regeneration. MS medium supplemented with 2,4-D in five concentrations (0.0, 0.1, 0.2, 0.3 and 0.4 mg/L) were used for the induction of embryogenic callus from *S. rebaudiana* explants. Then the resulted embryogenic callus was subcultured onto MS media containing various concentrations of BAP (0.0, 1.0, 2.0 and 3.0 mg/L), Kn (0.0, 5.0, 10.0 and 15.0 mg/L) and NAA (0.0, 0.1, 0.2 and 0.3 mg/L) for the induction of somatic embryo. Various concentrations of NAA (0.0, 0.1, 0.2 and 0.3 mg/L) and BAP (0.0, 1.0, 2.0 and 3.0 mg/L) were combinedly used for the development of multiple shoots from somatic embryo. MS media supplemented with various concentrations of IBA (0.0, 0.1, 0.2, 0.3 and 0.4 mg/L) were used for the induction of root from multiple shoots.

All the data were recorded at 14, 21 and 28 days after culture.

At zero concentration of 2,4-D, embryogenic callus formation was limited to all explants. However, maximum embryogenic callus (26.38%) was produced at 14 days after inoculation of 2,4-D (0.4 mg/L) in dark phase and the amount of callus was increased day by day. The maximum embryogenic callus 48.50% and 72.15% were found at 21 and 28 days after transfer / inoculation, respectively in dark phase. On the other hand, at 0.4 mg/L of 2,4-D; maximum embryogenic callus 23.11%, 45.13% and 59.90% was produced at 14, 21 and 28 days after inoculation, respectively in light phase.

Kn, at 5.0 mg/L was the best concentration for somatic embryo induction, followed by 15.0 mg/L compared to others. BAP at 1.0 mg/L was the best concentration for producing 18.59%, 37.92% and 40.63% somatic embryos at 14, 21 and 28 days after transfer of callus, respectively followed by 2.0 mg/L BAP than the others. On the other hand, NAA at 0.2 mg/L performed best to induce, 15.54%, 31.25% and 36.04% somatic embryos at 14, 21 and 28 days after transfer of callus, respectively. Further, 0.3 mg/L NAA was the better concentration than the others.

For plant regeneration, NAA and BAP were combinedly used in MS media. Among all the concentrations 0.1 mg/L NAA + 2.0 mg/L BAP was the best. Further 0.0 mg/L NAA+3.0 mg/L BAP, 0.1 mg/L NAA + 3.0 mg/L BAP, 0.2 mg/L NAA + 2.0 mg/L BAP, 0.3 mg/L NAA + 3.0 mg/L BAP and 0.3 mg/L NAA + 3.0 mg/L BAP were good concentrations for plant regeneration.

For root induction, among the different concentrations, IBA at 0.2 mg/L was the best for root number and 0.1 mg/L IBA was statistically similar. The same concentration (0.2 mg/L IBA) was found the best for root length and 0.1 mg/L IBA was statistically similar in this case.

The following conclusions could be drawn from the present investigation:

i) MS media supplemented with 0.4 mg/L 2,4-D was superior to others for callus induction.

ii) Dark phase was better for callus induction than light phase.

iii) Twenty eight days after inoculation was the best for getting highest amount of callus.

iv) MS media supplemented with 5.0 mg/L Kn was the best concentration for somatic embryo induction.

v) MS media supplemented with 0.1 mg/L NAA + 2.0 mg/L BAP was the best for plantlet regeneration.

vi) MS media supplemented with 0.2 mg/L IBA was the best for root induction.

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CHAPTER 6

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Appendices

APPENDICES

Appendix 1. Analysis of variance of the data of callus induction in dark phase and light phase at 14, 21 and 28 days after transfer on MS media supplemented with 2,4-D at five concentrations (0.0, 0.1, 0.2, 0.3 and 0.4 mg/L)

Source of variation	Degrees of freedom	Mean squares					
		Dark phase			Light phase		
		14 DAT	21 DAT	28 DAT	14 DAT	21 DAT	28 DAT
Treatment	4	355.69**	998.51**	2261.07**	303.44**	930.17**	2068.56**
Error	15	0.582	1.394	2.343	1.022	3.280	2.446

** Indicates significant at 1% level of significance

Appendix 2. Analysis of variance of the data of somatic embryo induction at 14, 21 and 28 days after transfer on MS media supplemented with NAA (0.0, 0.1, 0.2 and 0.3 mg/L), Kn (0.0, 5.0, 10.0 and 15.0 mg/L) and BAP (0.0, 1.0, 2.0 and 3.0 mg/L)

Source of variation	Degrees of freedom	Mean squares		
		14 DAT	21 DAT	28 DAT
Factor A	2	119.765**	80.131**	79.349**
Factor B	3	953.481**	2170.064**	3183.663**
AB	6	56.482**	147.838**	425.383**
Error	36	0.676	1.485	1.887

** Indicates significant at 1% level of significance

Here, Factor A – Hormones and Factor B – Concentrations of hormones

Appendix 3. Analysis of variance of the data of plant regenerations at 14, 21 and 28 days after transfer on MS media supplemented with NAA (0.0, 0.1, 0.2 and 0.3 mg/L) and BAP (0.0, 1.0, 2.0 and 3.0 mg/L)

Source of variation	Degrees of freedom	Mean squares		
		14 DAT	21 DAT	28 DAT
Factor A	3	25.631**	63.443**	410.106**
Factor B	3	335.789**	646.980**	1763.905**
AB	9	19.624**	72.965**	178.845**
Error	48	0.506	1.146	1.130

** Indicates significant at 1% level of significance

Here, Factor A – Concentrations of NAA and Factor B – Concentrations of BAP

Appendix 4. Analysis of variance of the data of root number and root length on MS media supplemented with IBA at five concentrations (0.0, 0.1, 0.2, 0.3 and 0.4 mg/L)

Source of variation	Degrees of freedom	Mean squares	
		Root number	Root length
Treatment	4	7.453**	3.324**
Error	15	0.304	0.373

** Indicates significant at 1% level of significance