In vitro propagation of *Stevia rebaudiana*

By

Rebeka Parveen
Reg. No. 03-01156

A Thesis

Submitted to Dept. of Horticulture and Post harvest Technology,
Sher-e-Bangla Agricultural University, Dhaka
in partial fulfillment of the requirements
for the degree
of

MASTER OF SCIENCE (MS)

IN

HORTICULTURE

Semester-June, 2008

Approved by:

Prof. M.A. Mannan Miah
Dept. of Horticulture & Post harvest Technology
Sher-e-Bangla Agricultural University
Supervisor

Dr. Ather-uz-Zaman
Head of Operation, Proshika
Plant Tissue Culture Center Trust
Co-Supervisor

Prof. Md. Ruhul Amin
Chairman
Examination Committee

Sher-e-Bangla Agricultural University

Sher-e-Bangla Nagar, Dhaka 1207

Sher-e-Bangla Nagar
Dhaka-1207
Tel : 9144270-8 Ext.-240

CERTIFICATE

This is to certify that the thesis entitled, " ***In vitro propagation of Stevia rebaudiana Bertony***". *submitted to the Dept. Horticulture & Postharvest Technology 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* ***Rebeka Parveen, Registration No. 03-01156*** *under my supervision and guidance. No part of this thesis has been submitted for any other degree or diploma.*

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

Dated: 30-06-08
Dhaka, Bangladesh

(Prof. **M.A. Mannan Miah**)
Dept. of Horticulture & Postharvest Technology
Sher-e-Bangla Agricultural University
Supervisor

Dedicated
to
my beloved parents

ACKNOWLEDGEMENT

All the praises belong to God Almighty, the most Gracious, Who enabled author with His blessings to undertake and complete this thesis and finally leading towards the fulfillment of the degree of Master of Science.

She takes this opportunity to express her gratitude and honor to her respectable and honorable Supervisor, Professor M. A. Mannan Miah, Department of Horticulture and Post harvest Technology, Sher-e-Bangla Agricultural University for encouraging her and giving her the opportunity to complete this interesting research work.

Profound appreciation, immense indebtedness and a lot of thanks to her honorable Co-Supervisor, Dr.Ather-uz-Zaman, Head of Operation, Proshika Plant Tissue Culture Center Trust for his kind cooperation, dynamic and constant guidance all through the research period and for photography.

She would like to express her cordial indebtedness and gratitude to all of the teachers of Department of Horticulture and Post harvest Technology ,Sher-e-Bangla Agricultural University, Mr. Kayes Samim, Agriculturist; Mr. Liakot Ali, Sr. Acc. Coordinator; Mr. Ripon, Senior Technician; Mr. Sushanto, Senior Technician; Mr. Israfil, Senior Technician and other technicians, staff and workers of Plant Tissue Culture Center Trust, Ipshita, Fariza, Muzahid, Zobaida, Sifat, Tithy, Sadek, Joyonto, Nafiur and all of her friends for their hardwork and cooperation from the beginning to the end the research work.

The Author

In vitro propagation of *Stevia rebaudiana* Bertoni

By

Rebeka Parveen

ABSTRACT

The experiment was conducted at the Plant Tissue Culture Center Trust Laboratory of Proshika Plant Tissue Culture Trust (PPTCC), Koitta, Manikgonj, Bangladesh from October, 2007 to June, 2008. For shoot proliferation, MS (Murashige and Skoog) and White's media supplented with four PGRs (BAP,Kn, NAA,ADS) were used singly at five concentrations (0.1, 1.0, 1.5, 2.0, 2.5 mg/L). BAP 2mg/L and Kn 1 and 2 mg/L were used in MS and White's media for synergism. PGRs and organic supplement viz., BAP, Kn, NAA, IB A, IAA and orange and tomato juice were added in Linsmiaier and Skoog (LS) and Phytamax (PM) media. For callus induction, 2,4-D 2mg/L was added in MS and White's media .For root formation, MS, LS, PM and White's media with IBA 2mg/L was used. For plantlet etablishment, the proportion of soil, sand and fertilizer /cowdung/poultry litter were used in 1:1:1. MS media with 2.5 mg/L was the best shoot proliferation using single PGR For, combined effect of PGR and organic supplement, best result was found with PM media supplented with IBA 0.5 mg/L. For synergism, best result was found with MS media supplemented with BAP mg/L with Kn 1 mg/L. Maximum root formation was observed with MS media with IBA 1 mg/L. MS media with 2,4-D 2mg/L was the best for callus induction. Combination of garden soil, sand and cowdung (1:1:1) was the best plantlet establishment comparing other treatments.

TABLE OF CONTENTS

CHAPTER		Page
	ACKNOWLEDGEMENTS	**i**
	ABSTRACT	**iii**
	TABLE OF CONTENTS	**iv**
	LIST OF TABLES	**v**
	LIST OF FIGURES	**vi**
	LIST OF ABBREVIATIONS	**vii**
1.	**INTRODUCTION**	**1**
2.	**REVIEW OF LITERATURE**	4
3.	MATERIALS AND METHOD	18
4.	RESULTS AND DISCUSSION	29
	4.1. Experiments with of BAP, NAA, Kn and ADS in MS medium	29
	4.1.1. Effect of BAP on multiple shoot proliferation	29
	4.1.2. Effect of NAA on shoot proliferation	32
	4.1.3. Effect of Kn on shoot proliferation	35
	4.1.4. Effect of ADS on shoot proliferation	**36**
	4.2. Study of BAP, NAA, Kn, ADS in White's media.	37
	4.2.1. Effect of BAP on shoot proliferation	37
	4.2.2.Effect of NAA on shoot proliferation and subsequent rooting	38
	4.2.3.Effect of Kn on shoot proliferation and subsequent root induction	**39**
	4.2.4.Effect of ADS on shoot proliferation	42
	4.3. Effect of 2,4-D on callus induction in MS and White's media	43
	4.4.Effect of IBA on root induction in different media	44
	4.5.Combined effect of organic supplements and PGRs on shoot proliferation in different media	48

CHAPTER		Page
	4.6.Interaction effect of different PGRs, their concentrations and media on shoot proliferation	51
	4.6.1.Effect of different concentrations	51
	4.6.2.Effect of different PGRs	**51**
	4.6.3.Effect of different media	52
	4.7. Synergistic effect of BAP and Kn	53
	4.8.Transfer of plantlets to soil and post transplantation observation	54
5.	**SUMMARY AND CONCLUSIONS**	**58**
	REFERENCES	**61**
	APPENDICES	**68**

LIST OF TABLES

	TITLE	Page
Table 1	Effect of different concentrations of BAP in MS medium on shoot proliferation and callus induction from shoot tip and nodal explans	30
Table 2	Effect of different concentrations of NAA in MS medium on shoot proliferation from shoot tip and nodal explant	32
Table 3	Effect of different concentrations of Kn in MS media on shoot proliferation from shoot tip and nodal explant	35
Table 4	Effect of different concentrations of ADS in MS media on shoot proliferation from shoot tip and nodal explant	36
Table 5	Effect of different concentrations of BAP in Whites's medium on shoot proliferation from shoot tip and nodal explants	38
Table 6	Effect of different concentrations of NAA in White's medium on shoot proliferation and rooting from shoot tip and nodal explant	39
Table 7	Effect of different concentrations of Kn in White's medium on shoot proliferation and rooting from shoot tip and nodal explant	40
Table 8	Effect of different concentrations of ADS in Whites's media on shoot proliferation from shoot tip and nodal explant	42
Table 9	Callus induction using 2,4-D in different media	43
Table 10	Formation of roots in response to IBA (1mg/l) in different media at 15 DAI	44
Table 11	Combined effect of PGRs and organic supplements on shoot proliferation in different media	48
Table 12	Effect of different PGRs, their concentrations and media on shoot proliferation	52
Table 13	Synergistic effect of BAP and Kn	54
Table 14	Growth observation of plantlets transplanted in pots	55

LIST OF FIGURES

	TITLE	Page
Figure 1	Effect of BAP 2.0mg/l on multiple shoot proliferation in MS medium	31
Figure 2	Effect of NAA 2.5 mg/l on shoot proliferation in MS medium	34
Figure 3	Effect of NAA 2.5 mg/L on root induction in MS medium	34
Figure 4	Effect of Kn 2.0 mg/L on shoot proliferation in White's medium	41
Figure 5	Effect of Kn 1.5 mg/L on shoot proliferation in White's medium	41
Figure 6	Effect of 2,4-D 2.0 mg/L on callus induction in MS medium	46
Figure 7	Effect of IBA 1.0 mg/L on root induction in liquid MS medium	47
Figure 8	Shoot proliferation LS+ 1 mg/L BAP+0.1 mg/L KIN+4mg/L Tomato	50
Figure 9	Plants grown in soil supplemented with poultry litter	57
Figure 10	Plants grown in soil supplemented with cowdung	57
Figure 11	Plants grown in soil without supplementation	57
Figure 12	Plants grown in soil supplemented with chemical fertilizers (NPK)	57

LIST OF APPENDICES

	TITLE	Page
Appendix I.	Preparation of MS medium	69
Appendix II	Preparation of White's medium	70
Appendix III	Preparation of Phytamax medium	71
Appendix IV	Preparation of Linsmiaier and Skoog medium	72

LIST OF ABBREVIATION

et al	*et alia aleii*(Latin) = and other people
viz	videlicet = namely
uM	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
PM	Phytamax
LS	Linsmere and Skoog
WH	White's
N	Normal
Gm/L	Gram (s) per litre
ml	mili litre (s)
mm	mili metere (s)
cm	centi metere (s)
^{o}c	Degree Celsius
h	hour (s)
2,4-D	Dichlorophenoxy acitic acid
Kn	Kinetin
IAA	Indole 3- acitic acid
IBA	Indole 3- butyric acid
ADS	Adenine sulfate
BAP	6- benzyl amino purine
NAA	∝- naphthalene acetic acid
G	gram (s)
Kg	Kilogram (s)
DAI	Days After Inoculation

Chapter 1

Introduction

CHAPTER 1

INTRODUCTION

The scientific name of stevia is *Stevia rebaudiana* Bertoni. It is a perennial herb belongs to the Asteraceae family. M. S. Bertoni, in the late 1800s, was the first European to document stevia. In 1931, French chemists extracted stevioside from the herb in the form of an intensely sweet, white crystalline compound (Jenny Hawke, 2002). This plant is a native of Paraguay and has been introduced into Japan, America, India, etc. In India, it is being cultivated on a large scale in Bangalore and Madhya Pradesh, etc (Ferri *et al.*, 2006).

Stevioside is regenerated as a valuable natural sweetening agent because of its relatively good taste and chemical stability (Yamazaki *et al.*, 1991). Stevia rebaudiana produces stevioside which is 300 times sweeter than sugar (Pude, 2005). Eight type of steviolglycoside are found in leaves viz., rebaudoside A, rebaudoside B, rebaudoside C (dulcoside), rebaudoside D, rebaudoside E, steviolbioside and dulcoside A. The four major sweeteners are stevioside , rebaudioside A, rebaudioside C and dulcoside A (Kinghorn,1992). The antihypertensive effect of crude stevioside obtained from the leaves of *Stevia rebaudiana* Bertoni (Compositae) on previously untreated mild hypertensive patients was examined (Geuns, 2003). It is considered a safe herb as it has no side effects and is undoubtedly consumable by the diabetic patients because it does not contain calories (Ferr *et al.*, 2006).

Stevia is new cultivated plant in Bangladesh. Broad spectrum cultivation of stevia is specially done by BRAC biotechnology division. In 2008, the total land area used for stevia cultivation was 1.5 acres and total dry leaf production was 500 kg. In 2007 and 2006, it was 150kg and 9kg respectively. The target of dry leaf production for 2009 is1000kg (BRAC report, 2009).

Shootlets were regenerated from nodal explants of *Stevia rebaudiana* Bertoni through axillary shoot proliferation (Ahmed, 2007).Somatic embryo

Under cultivation, Stevia can be propagated by seed and by vegetative propagation. Plant propagation by seed is not very efficient because of low seed fertility (Carneiro *et al.*, 1986). Oddone (1997) considers stevia to be self incompatible and inspect pollinated. Further, he considers "clear" seeds to be infertile. Vegetative propagation techniques, such as clump division, multiplication by cutting and micro propagation are in usual practice and micro propagation (Tamura *et al.*, 1984) 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.

Shoot apex, nodal, and leaf explants of *Stevia rebaudiana* can regenerate shoots from *in vitro* culture (Sivaram L. *et al.*, 2003) . *In vitro* propagation of stevia can be attemped in two methods .The first methods is 'Direct in vitro propagation' by multiple shoot regeneration from the explants (shoot tip and nodal segments) followed by rootin of multiple shoot where second method is 'Indirect *in vitro* propagation' by callus induction on the explants (leaf disc ,nodal segment internodal segments) followed by shooting and rooting of callus.

Objectives

1. To standardize the suitable media for *in vitro* shoot proliferation and root induction

2. To develop a micropropagation system to regenerate plantlets round the year

3. To find out optimum fertilization condition for it's establishment, better growth and development

Chapter 2

Review of Literature

CHAPTER 2

REVIEW OF LITERATURE

Mausumi Debnath (2008) said that Murashige and Skoog (MS) medium supplemented with 2.0 mg/L 6-benzyl amino purine and 1.13 mg/L indole-3- acetic acid in combination were found to be most effective in inducing bud break and growth, and in initiating multiple shoot proliferation at the rate of 39 microshoots per nodal explant after 30 day of culture. By repeated subculturing a high-frequency multiplication rate was established for production of elite lines of *Stevia rebaudiana*. Elongated shoots were transferred to rooting medium. MS medium supplemented with 2.0 mg/L indole-3-butyric acid was found to be best for rooting.

M.B.Ahmed (2007) found that shootlets were regenerated from nodal explants of *Stevia rebaudiana* Bertoni through axillary shoot proliferation. The induction of multiple shoots from nodal segments was the highest in MS medium supplemented with 1.5 mg lG BA + 0.5 mg lG Kn. For rooting different concentrations of IBA, NAA and IAA were used and highest rooting percentage (97.66%) was recorded on MS medium with 0.1 mg lG IAA. The rooted plantlets were hardened and successfully established in soil.

Aparajita *et al.* (2007) said that, procedure for micropropagation of *Stevia rebaudiana* Bertoni, containing stevioside, a natural noncaloric sweetner, has been developed by using nodal segments as explant. Higher proliferation of shoots and multiplication was obtained on Murashige and Skoog basal medium (MS) supplemented with 1.0 mg/L indoleacetic acid (IAA) plus 10.0 mg/L kinetin and 30.0 mg/L adenine sulphate. Sprouting of 90% of the axillary buds was observed within 4 weeks of inoculation, producing >10.0 shoots per explant within 12 weeks. Profuse roots were induced from 90% of the regenerated shoots within 4 weeks of inoculation on half strength MS

solid medium supplemented with 1.0 mg/L IAA. High survival rate, >60%, was obtained when the plantlets were transferred to field conditions. The heterogenic nature of *S. rebaudiana* necessitates establishment of protocol for every genotype independently.

Huda *et al.* (2007) said that, *In vitro* morphogenic responses of different explants of Stevia (*Stevia rebaudiana* Bert.) by Leaf segments, internode and nodal segments of Stevia were cultured on MS medium supplemented with varying concentrations (1, 3, 5 and 7 mg/L) of growth regulators (2,4-D, BAP and NAA) along with coconut water for observe morphogenic responses (mainly formation of callus). Nodal segment initiated callus earlier than leaf segments and internode explant. Higher amount of callus were obtained from leaf segments than internode and nodal segment. Interaction effects of explant and growth regulators were significant for days of callus initiation, fresh weight and dry weight of the callus per culture. Among the twelve treatments the highest amount of callus was obtained in MS medium supplemented with 7 mg/L BAP followed by 3 and 5 mg/L NAA, respectively. On the other hand, 7 mg L-1 2,4-D showed lowest performance followed by 5 mg L-1 2,4-D and 5 mg/L BAP, respectively among the twelve treatments.

Koppad *et al.*(2006) conducted a study in Sirsi, Karnataka, India to evaluate the effects of IAA (100, 300 and 500 pppm) and coumarin (100, 300 and 500 ppm) on the propagation of stem cuttings of Stevia rebaudiana. IAA at 500 ppm and coumarin at 300 ppm were identified as the best treatments, recording the highest values for number of roots and root length.

Himanshu *et al.* (2006) said that, MS basal medium supplemented with 13.5 micro M benzyladenine + 8.0 micro M NAA showed best performance compared to 13.3 micro M benzyladenine alone with respect to bud breaking in *S. rebaudiana*, while combination of 13.3 micro M benzyladenine + 61.8 micro M $AdSO_4$ and 13.5 micro

M benzyladenine + 8.0 micro M NAA in MS basal medium showed enhanced shoot multiplication. NAA at 16.1 micro M + 10.7 micro M benzyladenine showed significant enhancement in the induction of rooting compared to 16.1 micro M IAA alone. The best callusing was observed in the media containing 10.7 micro M NAA + 8.8 micro M benzyladenine.

Kuntal-Das *et al.* (2006) said that *S. rebaudiana* callus was grown under aseptic environment on MS medium (1/2, 1 and 2 strength) supplemented with plant growth regulators (IAA, NAA, IBA, 2,4-D, kinetin and BAP [benzyladenine]). Extensive callus initiation was observed with 2,4-D at 1.0 mg/L + kinetin at 0.2 mg/L with callus growth values of 2.25 and 2.20 in half and single strength media, respectively. Callus was best maintained with NAA at 0.1 mg/L and BAP at 2.0 mg/L with callus growth values of 4.30 and 4.25 cm in half and single strength MS media, respectively.

Uddin *et al.* (2006) conducted an experiment on *in vitro* culture of *Stevia rebaudiana* Bertony, an important non-caloric sweetening herb to explore its potential for micro-propagation. Leaf, nodal and inter-nodal segments of the selected herb as explant were cultured on MS medium containing 2,4-D at 2, 3, 4 and 5 mg/L for callus induction. Inter-nodal segments initiated callus earlier than node and leaf. The highest amount of callus was found in MS medium with 3.0 mg /L 2,4-D and MS medium with 5.0 mg/L 2,4-D gave the poorest callus.

Kuntal Das *et al.*(2006) conducted a greenhouse experiment at the Indian Institute of Horticultural Research (IIHR), Bangalore, to study the effect of combined applications of nitrogen (N), phosphorous (P) and potassium (K) fertilizers on their availability in soils in relation to their contents in the Stevia plant. The results show that the amount of available N, P and K in soil has been found to be increased initially up to 45 days and thereafter, the amount of the same content decreased with the

progress of plant growth up to 60 days irrespective of treatments. However, the magnitudes of such increases in N, P and K contents both in soils and plants have also been enhanced with the simultaneous application of N, P and K (40:20:30) kg/ha over that of their corresponding individual applications.

Kabir (2005) studied two methods *In vitro* propagation of stevia. The first method was 'Direct *in vitro* propagation' by multiple shoot regeneration from the explants (shoot tip and nodal segments) followed by rooting of multiple shoot where second method was 'Indirect *in vitro* propagation' by callus induction on the explants(leaf disk, nodal segmemt and internodal segment) followed by shooting and rooting of 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, 3.0 mg/L). Auxin viz .NAA and IBA were used for root induction for micro cuttings 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, 7.0 mg/L) were used for callus induction and then subsequently sub cultured onto MS medium containing 1.0 mg /L BAP and 1.5mg/L NAA for shoot and induction of 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 was superior to inter and nodal segment .MS media supplemented with BAP (7.0 mg/L) was best medium for the callus formation.

Kuntal Das *et al.* (2005) studied *In vitro* methods for production of stevioside from *Stevia rebaudiana* .The callus culture of leaves of *S. rebaudiana* was initiated and maintained on different strengths (half and full) of MS medium supplemented with various plant growth regulators. The initiation of callus was achieved after supplementing the various concentrations and combinations of auxins, i.e. IAA, IBA, NAA and 2,4-D, each at 1 mg/L and cytokinin kinetin at 0.1, 0.2 and 0.3 mg/l. The maintenance of independent callus was carried out on different strengths of MS medium supplemented with IAA, NAA and 2,4-D, each at 0.1 mg/L, and kinetin and BAP [benzyladenine], at 1.0 and 2.0 mg/L, at different combinations. The callus was maintained up to 3 cycles by transferring the cultures to fresh medium after every 2-3 weeks. The half strength medium supplemented with 2,4-D (1.0 mg/L) and kinetin (0.2 mg/L) was the best for callus initiation and the NAA (0.1 mg/L) and BAP (2.0 mg/L) combination was the best for maintenance.

Sumathi *et al.*(2005) observed that the highest amount of callus was found in MS medium with 3.0 mg/L 2,4-D and MS medium with 5.0 mg/L 2,4-D gave the poorest callus.

Smitha *et al.* (2005) developed a procedure for the rapid clonal propagation of *S. rebaudiana* explants such as axillary shoot buds, terminal shoot buds and leaf segments. Multiple shoots were induced from shoot buds on a modified MS medium supplemented with 1.0 mg/L benzyladenine (BA). Multiplication rate of up to 1:25 was observed. Incorporation of 0.05 mg kinetin/litre enhanced the production of dark green healthy shoots. Successful rooting (>90%) was recorded in modified MS medium supplemented with 1.5 mg/L IBA. The roots appeared normal and healthy. The plantlets were hardened in sterilized sand and were established *ex vitro* with more than 90% success. In addition, direct plantlet regeneration was also induced from leaf explants on modified MS medium supplemented with 1.0 mg BA/litre and 0.1 mg NAA/litre.

Islam (2004) reviewed shoot tip, nodal segment and leaf base with petiol of *S. rebaudiana* were cultured on MS media supplemented with various concentrations of BA for shoot production. Nodal segment initiated shoot earlier than shoot tip explant. Higher number of shoot per culture and micro cuttings were obtained from shoot tip explant and 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 MS medium with 3.0mg/L BA and 2.0 mg/L BA respectively. Interaction effect of explant and shooting media were significant for days offshoot initiation ,number of shoot per culture 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).

Bondarev *et al.*(2003) showed the effects of nutrient medium composition on development of *Stevia rebaudiana* shoots cultivated in the roller bioreactor and their production of steviol glycosides . Addition of 0.1 mg/L BA or 6-benzylaminopurine (benzyladenine) (BA) together with alpha -naphthaleneacetic acid resulted in an 1.5-fold increase in the number of shoots. However, the shoots grown on the BA-supplied medium displayed a strong inhibition of the development of their root system. When the medium was supplied with gibberellic acid, lengthening of shoots and roots of Stevia were observed. All the plant growth regulators used strongly inhibited production of SGs. The changes in nutrition medium composition had practically no effect on the ratio of individual glycosides in Stevia leaves. Shoot apex, nodal, and leaf explants of *Stevia rebaudiana* can regenerate shoots when cultured on MS medium supplemented with benzyladenine (BA; 8.87 micro M) and IAA (5.71 micro M). Rooting of the *in vitro*-derived shoots was achieved following subculture onto auxin-containing medium.

Moriniet *et al.* (2003) said that, growth chamber temperature was 24+or-1 degrees C, photoperiod was 16-h and light intensity was 45+or-5 micro mol m-2 s-1. Shoot rooting response was evaluated by IBA at 0.5 mg/L and 0.1 mg/L. Susceptibility to tissue vitrification, very small leaves and thin and petiolated stem apical portions were associated particularly with 6-benzylaminopurine, in most genotypes. With kinetin, symptoms were attenuated but did not disappear completely. Among the genotypes tested, shoot proliferation ranged from about 1:4 to 1:9 and rooting from 65 to 92%. Fairly severe problems were encountered in plantlet acclimatization, with highest survival values not exceeding 80%. Micropropagation efficiency did not appear to be very satisfactory but the experimental procedure demonstrated the possibility of quickly propagating plants of Stevia to be used for cultivation and/or propagating selected genotypes characterized by superior biological and agronomic traits and/or higher glucoside content, to be used as mother plants to provide material for other propagation techniques.

Maharik *et al.* (2003) reviewed that *S. rebaudiana* seeds were germinated in Murashige and Skoog's (MS) medium containing 2% sucrose and 0.7% agar. Twelve of the 15 seeds germinated and produced seedlings with 3-4 leaf pairs at 12-16 days after sowing. Nodal segments (0.6-1.0 cm long with one leaf pair each) were obtained from seedlings and cultured in MS basal medium supplemented with 6-benzyladenine (benzyladenine), NAA and IBA at various concentrations (2+2+0, 2+1+0, 4+2+0, 5+3+0, 6+3+0, 6+2+0, 5+2+0 or 5+2+0 mg), along with 3% sucrose and 0.7% agar. Healthy shoots approximately 2.3 cm long were isolated from each culture and inoculated on half-strength MS medium supplemented with IBA, 20% sucrose, 0.1% activated charcoal and 0.7% agar. Rooting was induced in jars containing 50 ml of the medium. The medium containing 5 mg IBA + 2 mg NAA resulted in the highest number of healthy shoots (46.9 shoots per culture). When benzyladenine was

combined with IAA, callus initiation occurred. Reducing the concentrations of benzyladenine and NAA to 4 and 2 mg/L, respectively, resulted in callus initiation and poor shoot multiplication. As the IBA concentration increased, callusing increased at the base of elongating shoots, followed by delayed and poor rooting. The addition of activated charcoal to the rooting media improved root development. Rooted plantlets were transplanted into pots containing a mixture of vermiculite and peat moss (1:5 v/v) and acclimatized in the greenhouse for 30 days.

Slavova *et al.*(2003) said that, shoot and stem segments of *S. rebaudiana* were cultured in 2 nutrient media, one with BAP [benzyladenine] and the other with NAA for rooting. Benzymidazol at 0.1, 0.2, 1.0, 1.5, 2.0 or 3.0 mg/L was incorporated after the removal of BAP and NAA from both media, except for one treatment in which 0.3 mg benzymidazol + 0.1 mg/L NAA was evaluated. Benzymidazol exhibited an auxin-like effect (i.e. stimulation of rooting) on plants. When benzymidazol was applied singly, 0.2 mg/L was optimum, resulting in 95% rooting. The 2-4 roots per plant formed at this concentration were stable and well developed, unlike those that were produced with NAA, which were easily severed when removed from the nutrient medium. The highest rooting percentage (99%) was obtained with 0.3 mg benzymidazol + 0.1 mg/L NAA.

Sivaram L.*et al.*(2003) said that, Shoot apex, nodal, and leaf explants of *Stevia rebaudiana* Bertoni can regenerate shoots when cultured on Murashige and Skoog (MS) medium supplemented with 6-benzyladenine (BA; 8.87 μ*M*) and indole-3-acetic acid (5.71 μ*M*). 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..

Zbughin *et al*. (2002) presented a study on the effects of IAA and BA in the MS culture medium on the propagation of *S. rebaudiana* from meristems by. Mathematical models are presented to estimate the changes in different characteristics (number of leaflets, height of stemlets, number of axillary buds, number of secondary copse, and number of basal rootlets) of *S. rebaudiana* under different IAA and BA treatments.

Bondarev *et al*.(1998) observed that the effect of growth regulators such as NAA, 2,4-D, Kn and BA on the callusogenesis and growth of *Stevia rebaudiana* (a perennial grass which contains dipterpenoid glucosides 300times as sweet as sucrose) cultured cells. A significant retardation of callus growth and complete imhibition of organogenesis took place at 2,4-D concentrations ranging from 0.2-1.0 mg/L).

Bespalhok *et al.* (1997) said that, 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 kinetin (0-9.29 μM).On 9.05 μM 2,4-D supplemented medium without kinetin, maximum embryogenic callus formation occurred. On 18.10μM 2,4-D supplemented medium, the best results were obtained with 2.32μM kinetin. Callus formation started at the base of the corolla and ovaries.

Constantinovici *et al*.(1997) micropropagated *S. rebaudiana* from shoot apex or internodal explants on 3 different multiplication media and 2 rooting media. Shoot apices performed better as explants than nodal stem sections. Benzyladenine was more effective than kinetin in the multiplication medium. Roots emerged from the internodes on either of the rooting media (growth regulator-free or containing kinetin). Rooting was essential before plantlets were transferred ex vitro.

Bespalhok *et al*.,(1997) studied that Somatic embryos were induced when leaves were cultured in vitro on MS medium supplemented with 2,4-D (10 or 25 μM).The embryo appeared to be formed directly without intermediate callus development .somatic embryo failed to mature developed roots but not shoots when transferred to MS medium without growth regulators and with a low (30 gm/L) concentration o sucrose.

Kornilova *et al*.(1996) developed a laboratory procedure for the clonal micropropagation of *S. rebaudiana*, using stem segments with 2 axillary buds. Use of MS medium without growth regulators gave results comparable to those obtained with growth regulators (BA + NAA and kinetin + 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.

Sritongkum (1995) developed the procedures for micropropagation primary leaf and shoot tip of *S. rebaudiana* Bertony. The optimal condition from primary leaf were observed on MS medium supplemented with 2.0mg/L NAA and 2.0 mg/L IBA .The optimal condition 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 formation 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.

Carvalho *et al.* (1995) said that, treatment with IBA promoted rooting and increased the number of roots produced in cuttings taken in August and September, whereas IAA and NAA did so only for cuttings taken in August. Percentage rooting was highest (80%) in cuttings taken in August and treated with IBA at 5 x 10-5 M. The treatments had no effect on percentage cutting survival after transplanting.

Kornienko *et al.*(1995) found the results of cultural trials with stevia [Stevia rebaudiana] in the Central Chernozem Zone of Russia are discussed. Preceding crops, soil cultivation, weed problems, nutrition, planting dates, spacing in relation to planting date, and growth regulator treatments were considered. The regulators, applied 30-40 days after planting, were particularly effective on unfertilized controls. PABK-DA (p-aminobenzoic acid) at 0.005% plus 80 kg N + 80 kg P_2O_5 + 80 kg K_2O/ha was the best treatment, producing 53.9 and 13.79 t/ha fresh and dry biomass, respectively. Higher NPK rates reduced yields

Bespalhok *et al.*(1992) revealed two long nodal d\segment were excised from adult *S. rebaudina* plant cultured for shoot proliferation on MS medium containing six levels of NH_4 NO_3, the fossard vitamins,11 µM BA, 3% sucrose and 1% agar. When shoots were about 5 cm long they were transferred fur rooting to full or half strength MS medium containing NAA at 0.0 to 10.0 µM. Lowering the NH_4 $NO3$ concentration in the multiplication medium 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_4 $NO3$ 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%.
Bespalhok *et al.* (1992) develops the procedures of excising two-cm-long nodal segments from adult *S. rebaudiana* plants and cultured for shoot proliferation on MS medium containing 6 levels of NH_4NO_3, De 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%.

Swanson *et al.* (1992) studied that leaf explants of *Stevia rebaudiana* were cultured in MS medium with vitamins, sucrose (30 g/litre) and agar (0.9% w/v) and supplemented with NAA (0.5 mg/L) and BA (0.5 mg/L). These conditions yielded friable callus cultures. Differentiation of the callus tissue was then achieved by eliminating the agar and modulating the medium's hormone concentrations. Thus, media containing increased auxin concentration (1.0 mg/L) and no cytokinin, or increased cytokinin (1.0 mg/L) and no auxin, yielded root or shoot cultures, respectively. Supplementation of the shoot medium with NAA (1.0 mg/ml) induced shoot cultures to grow roots thereby differentiating into rooted-shoot cultures.

Ferreira *et al.*(1988) described a method for producing and maintaining *Stevia rebaudiana* suspensions and regeneration of plants from calli derived from cell suspensions. Suspension cultures composed of isolated cells (ca. 10%) and cellular aggregates (5–100 cells) were obtained in 20–30 days by using friable callus as the initial inoculum in liquid media with BA (0.5 mg/l)+2,4-D (1.0 mg/l), and periodic filtering (100–500 µm sieves) with 6–7 days interval between subcultures. Cultures derived from actively growing calli are mainly diploid (2n=22) whereas those derived from senescent calli showed a wide variation in chromosome number (55–200). Shoot

elongation and further rooting of isolated shoots was better on LS medium devoid of growth regulators.

Angkapradipta *et al.* (1986) said that, seedlings in pots (one per pot of 5.5 kg soil) received a basal dressing of 200 g cattle manure and urea at 0-2.0 g, triple superphosphate at 0-0.25 g and potassium chloride at 0-0.20 g/pot. Cattle manure markedly improved soil fertility. Increasing rates of N increased plant N content, and reduced soil pH, K, Ca and Mg. The P and K fertilizers increased soil P and K contents but did not affect plant P and K contents. N at 1 g/pot significantly increased foliage fresh and dry matter production and the fresh weight of roots. A rate of 0.91 g urea per plant up to the first year of harvest is suggested.

Goenadi (1985) said that, in a glasshouse trial with pot-grown plants, the effects were compared of FYM at 0, 100 or 200 g/plant, NPK (28.75:9.38:10.00) at 0, 1.2 or 2.4 g/plant, and liquid organic fertilizer as a 200 ml solution at 0, 5 or 10% (v/v)/plant. Plant growth and leaf numbers/plant were increased by FYM and liquid fertilizer, the best calculated results being obtained at 175 g/plant and 6.25% liquid fertilizer/plant, respectively. NPK had no effect.

Buana *et al.* (1985) measured Plant height and leaf number weekly in *Stevia rebaudiana* cuttings fertilized with various levels of NPK, farmyard manure or a 'Super Mineral' fertilizer. Equations are presented relating growth during the first 4 weeks to the level and type of fertilizer applied.

TamuraY.*et al.* (1984) established clonal propagation of *Stevia rebaudiana* by culturing stem-tips with a few leaf primordia on an agar medium supplemented with a high concentration (10 mg/l) of kinetin. Innumerable shoots can be obtained by repeating the cycle of multiple-shoot formation from a single stem-tip of Stevia.

These shoots produce roots when transferred to a medium containing NAA (0.1 mg/l) without kinetin. The regenerated plantlets can be transplanted to soil.

Lee *et al*. (1980) studied that, nitrogen P_2O_5 and K_2O at rates of 0, 10 and 15 kg/10 a each, applied in various combinations did not affect *Stevia rebaudiana* plant height, number of branches or number of internodes.

Yang. *et al*. (1979) conducted an experiment and found that. Rapid plant regeneration of *S. rebaudiana* was achieved by culturing leaflets of 12 to 16-day-old seedlings on a modified Murashige and Skoog medium (MS). Multiple shoots were induced on MS supplemented with 2-10 mg/l BA. Roots were induced by sub culturing the regenerated shoots on the MS medium without hormones or on moistened vermiculite. Successful transferring of plantlets to soil was accomplished and production of flowering plants on a large scale was feasible.

Handro *et al*.(1977) observed that Leaf discs and 1-cm internodal stem segments cultured on synthetic media containing an auxin (IAA or 2,4-D) and a cytokinin (benzyladenine or kinetin) produced vigorous calli. Two-cm nodal stem segments grew well on a medium without hormones.

Murashige *et al*.(1962) said that, MS medium 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 shoot could be achieved following subculture onto auxin containing medium .

Chapter 3

Materials and Methods

CHAPTER 3

MATERIALS AND METHOD

The experiment was conducted at Laboratory of Proshika Plant Tissue Culture Center Trust (PPTCCT), Koitta , Manikgonj from October, 2007 – June, 2008. The materials and methods used in this investigation are described below.

3.1. Plant Materials

One month old *Stevia rebaudiana* growing in polybags were collected from BRAC nursery, Gazipur, Bangladesh. The plants were further allowed to grow at Proshika nursery for the ready explants sources. Leaf and nodal segments were used as explants. Leaf and node segments from plants were cut and rinsed in running water for 20 minutes. The explants were surface sterilized in laminar airflow cabinet and 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. Glassware and plastic ware

Test tubes (25 x 150 mm), conical flasks (125, 150, 250, 500, 1000 ml capacity), pipettes 2, 5 and 10 ml capacity), measuring cylinders, funnels, etc. used in various experiments were of Coming or Borosil make.

Locally manufactured glass jars with polypropylene screw caps were used as culture vessels. All glassware was cleaned using liquid soap and thoroughly washed in

running tap liter. Washed glassware was rinsed with distilled water and oven dried before use.

Culture tubes and flasks were plugged with absorbent cotton prior to autoclaving. Flasks and bottles containing infected cultures were decontaminated by sterilization for 1 hour, prior to washing.

Chaina made petri-plates (55 × 15 mm and 85 × 15mm) were also used in the studies. Autopipettes (0-20 μl, 20-200 μl and 200-1000 μl) and autoclavable micro-pipette tips (20, 200 and 1000 μl capacities) were used for accurate addition of fine chemicals in the medium.

3.3. Glassware sterilization

All the empty glassware to be used for media preparation (flasks, bottles, test tubes, pipette tips) and instruments needed for sterile dissection and subculturing (forceps, scalpel, and filter paper pads) were autoclaved at 121°C and 1.1 kg/cm^2 pressure for 1 hour.

3.4.Preparation of stock solution

The first step in the preparation of culture media was the preparation of stock solution. Various constituents of the medium were prepared into stock solutions for immediate use during the preparation of the medium. As different constituents were required in different concentrations to separate stock solution for macronutrients, micronutrients, vitamins, growth regulators etc were prepared separately and stored at refrigerator

- **Preparation of stock solution I (macronutrients)**

One litre stock solution I was made up to 50 times of the final concentration of the

medium. At first 600 ml of distilled water was taken in a 1500 ml beaker and 82.5 gm NH_4NO_3, 95 gm KNO_3, 8.5 gm KH_2PO_4 were added and dissolved by random stirring with a magnetic stirrer. Then distilled water was added to make the volume 1000 ml and stored in a reagent bottle refrigerator.

- **Stock solution II (macronutrients)**

One liter stock solution II was made up to 100 times of the final concentration of the medium. At tint 600 ml of distilled water was taken in a 1500 ml beaker and then 44 gm $CaCl_2.2H_2O$ weighed accurately and dissolved. Then distilled water was added to make the volume up to 1100 ml and stored in a reagent bottle in refrigerator.

- **Stock 5olutlon III (macronutrients)**

One liter stock solution III was made up to 200 times of the final concentration of the hum. At first 600 ml of distilled water was taken in a 1500 ml beaker and then 74 gm $MgSO_4.7H_2O$ weighed accurately and dissolved. Then distilled water was added to make the volume up to 1000 ml and stored in a reagent bottle in refrigerator.

- **Stock solution IV (micronutrients)**

One liter (v/v) stock solution ill was made up to 100 times of the final concentration of medium. At first 600 ml of distilled water was taken in a 1500 ml beaker and then 2.78 gm $FeSO_4$7H20 and 3.37 gm Na_2-EDTA. $2H_2O$ were weighed accurately and dissolved. Distilled water was then added to make the volume up to 1000 ml and stored in a reagent bottle in refrigerator.

- **Stock solution V (micronutrients)**

One litter stock solution V was made up to 200 times of the final concentration of the medium. At first 600 ml of distilled water was taken in a 1500 ml beaker and then 1.24 gm H_2BO_3 1.72 gm $ZnSO_4.7H_20$, 4.46 gm $MnSO_4.4H_20$ were weighed accurately

and dissolved. The distilled water was added to make the volume up to 1000 ml and stored in a reagent bottle in refrigerator.

- **Stock solution VI (micronutrients)**

One liter stock solution VI was made up to 100 times of the final concentration of the medium. At first 600 ml of distilled water was taken in a 1500 ml beaker and then 83 gm KI, 2.5gm $CuSO_4.5H_20$, and 25 gm $NaMoO_4.2H_20$, 2.5mg $CoCl_2.6H_20$ were weighed accurately and dissolved. Then distilled water was added to make the volume up to 1000 ml arid stored in a reagent bottle in refrigerator.

- **Stock solution VII (organic nutrients)**

One liter stock solution VII was made up to 100 times of the final concentration of the medium. At first 600 ml of distilled water was taken in a 1500 ml beaker and then 20 gm myoinosiol, 0.1 gm Nicotinic acid, 0.1 gm Pyridoxine HC1, 0.02 gm Thiamine HC1, 0.4 gm Glycine were weighed accurately and dissolved. Then distilled water was added to make the volume up in 1000ml and stored in refrigerator.

3.5. Stock solution of PGRs

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 acetic acid (IAA)
Indol-3 butyric acid (IBA)
∝- napthalene acetic acid (NAA)
2, 4-Dichiorophenoxy acetic acid (2,4-D)

Cytokinins

6-benzyl amino purine (BAP)
Kinetin (Kn)
Adenine sulfate (ADS)

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

Hormones (Solute)	Solvents
IBA	70% ethyl alcohol
NAA	0.1N NaOH
2,4-D	70% ethyl alcohol
BAP	0.5N HC1
Kn	0.5N HC1
ADS	0.5N HC1
IAA	0.5N HC1

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.

3.6. Media preparation

To prepare any of hormonal stock solution, 100 mg solid hormone was dissolved in 1 ml solved (0.1 N KOH). The mixture was then washed off with distilled water and collected in a measuring cylinder and was made up to the mark with further addition of distilled water. So the strength of the hormonal stock solution becomes 1.0 mg/ml for the preparation of 200 ml of any media following steps were followed:

i) The stock solution I, II, III, W, V, VI, VII were taken into a 500 ml conical flask in amounts of 4,2, 1,2, 1,0. 1 and 1 ml, respectively.
ii) Different required concentrations of hormonal supplements were added to this solution individually.
iii) 6 gm of sucrose for MS basal medium was dissolved in 200 ml mixed components.

iv) The whole mixture was then made up to 200 ml with further addition of distilled water.

v) pH of the medium was adjusted to 5.8.

To prepare solid medium, 0.6% agar was added to the solution and heated in microwave oven. Then the media was dispensed into test tubes in a volume of 15ml.

- **Callus induction medium**

MS (Murashige and Skoog, 1962) and White's media supplemented with 2,4-D (2.0 mg/L) was prepared for the callus induction from *S. rebaudiana* explants. After mixing all stock solutions 3% sugar was added for the MS and White's media preparation. Then the pH of the media was adjusted to 5.8. 0.6% agar was dissolved and the media was heated and dispensed in the jars and capped withpolypropylene screws. Jars containing media were autoclaved at 121°C at 1.1 Kg / cm^2 for 20 minutes.

- **Plantlet regeneration medium**

✓ MS and White's media supplemented with various concentrations of PGRs singly were added to prepare plantlet regeneration media. PGRs and their concentrations -

  - BAP - 0.5 mg/l, 1.0 mg/l, 1.5mg/l, 2.0 mg/l, 2.5mg/l
  - NAA - 0.5 mg/l, 1.0 mg/l, 1.5mg/l, 2.0 mg/l, 2.5mg/l
  - Kn - 0.5 mg/l, 1.0 mg/l, 1.5mg/l, 2.0 mg/l, 2.5mg/l
  - ADS - 0.5 mg/l, 1.0 mg/l, 1.5mg/l, 2.0 mg/l, 2.5mg/l

✓ For combined effect of PGRs, 2mg/l BAP and 1 and 2mg/l Kn were added in MS and White's media.

✓ For combined effect of PGRs and Organic supplements,

  - Media Usesd-LS, PM
  - PGR added -BAP, Kn, NAA, IBA, IAA
  - Organic supplements-Orange juice, Tomato juice

The 0.6% agar was dissolved and the media was heated and dispensed in the test tube and capped with aluminum foil. Jars containing media were autoclaved at 12 1°C at 1.1 Kg /cm^2 for 20 minutes.

- **Root growth medium**

$^1/_2$ MS (Murashige and Skoog, 1962) $^1/_2$ LS, $^1/_2$ PM and $^1/_2$ White's media supplemented with IBA 2mg/l were prepared for the induction of root from shoot tips and nodal explant. 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 and culture vessel as above and capped with aluminum foil. Jars containing media were autoclaved at 121°C at 1.1 Kg/cm^2 for 20 minutes.

3.7. Media sterilization

All nutrient media were autoclaved at 1.1 kg /cm^2 pressure at 121°C for 20 minutes. Heat labile chemicals were added in the required quantity, after filter sterilization, into the autoclaved media.

3.8. Culture environment

Cultures were incubated at 22±2°C with a photoperiod of 16 hrs at 3000 lux light mtensity of cool white fluorescent light.

3.9. Experimental design

The experiment was designed as 2 × 5 factorials in a Completely Randomized Design with 5 replication and 5 plantlets per replication.

3.10. Methods of scoring growth response

a) Percentage of explants responded

$$\% \text{ of explants responded} = \frac{\text{No. of explants responded}}{\text{Total no. of explants inoculated}} \times 100$$

b) Percentage of root Induction

$$\% \text{ of root induction} = \frac{\text{No. of root induction}}{\text{Total no. of shoot inculated}} \times 100$$

c) Average number of multiple shoot

$$\overline{X} = \frac{\sum Xi}{N}$$

Where, $\overline{X}$ = Average no. of multiple shoot

$\sum$ = Summation

Xi= Number of multiple shoots
N = Number of observations

37216 04-11-19
A. 95 13/09/09

d) Average length Of shoot

$$\overline{X} = \frac{\sum Xi}{N}$$

Where, $\overline{X}$ = Average length of shoot

$\sum$ = Summation

Xi = Length of shoots

N = Number of observations

e)Average length of root

$$\overline{X} = \frac{\sum Xi}{N}$$

Where, $\overline{X}$ = Average length of shoot

$\sum$ = Summation

Xi = Length of shoots

N = Number of observations

3.11.Data collection

Visual observation of culture was made every day. Data were recorded for percentage of explants responded, percentage of root Induction , average number of multiple shoot, average length of shoot , average number and length of root and callus induction . Except average number and length of root and callus induction, all data were recorded 15 days and 30 days after inoculation. The data for average number and length of root were recorded after 7 days of inoculation.

3.12. Data analysis

All the data were assessed by analysis of variance for factorial complete randomized design (CRD) using computer software SPSS Program (General linear Model-Univariate Analysis).

3.13. Hardening of plantlets

For hardening the *in vitro* generated plantlets were removed carefully and dipped in 0.2% Ridomil-100EC (antifungal) for few minutes. Finally the plantlets were tranferred 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 shades.

3.14. Pot preparation

The hardened plants were planted in earthen pots filled with different combinations of soil which were prepared using garden soil, poultry litter, cowdung and chemical fertilizers (Urea, TSP, MP). The proportion of garden soil, sand and poultry litter /cowdung /chemical fertilizer was 1: 1: 1. Ten replications were done against each treatment or combination.

3.15. Transplantation of hardened plantlets

After 2-3 weeks of hardening of plantlets they become 7-10 inches in height and strong enough for plantation to soil under normal condition. Therefore, they were then transferred to earthen pots where they were grown. Care was taken of that roots did not get any damage during plantation. Watering and weeding were done in the normal course depending on the texture of the soil. Four soil combination were used .

Combination 1. Garden soil + sand (1:1)
Combination 2. Garden soil + sand + cowdung (1:1:1)
Combination 3. Garden soil + sand + poultry litter (1:1:1)
Combination 4. Garden sol + sand + chemical fertilizer (1:1:1)

Chapter 4

Results and Discussion

CHAPTER 4

RESULTS AND DISCUSSION

The results of the investigations have been presented and discussed in the forms of tables, and figures. The results and discussion of experiments are discussed below.

4.1. Experiments with of BAP, NAA, Kn and ADS in MS medium

41.1.Effect of BAP on multiple shoot proliferation

Shoot tips and nodal explants were cultured on MS medium supplemented with different concentration of BAP. Both 1.0 and 1.5 mg/l were proved as the best concentrations but BAP 2.0 mg/l revealed poor response. Kabir (2005) reported that shoot tip explants showed better response for shoot proliferation than nodal segments and MS medium in full and half strength were equally effective for shoot proliferation .The concentration used in the recent study were 0.5 mg/l, 1.0 mg/l, 1.5 mg/l, 2.0 mg/l and 2.5 mg/l and results are shown in table-1. The data were collected at 15 DAI and 30 DAI. Multiple shoots were proliferated in response to BAP. Smitha *et al.* (2005) observed that multiple shoots were induced from shoot buds on a modified MS medium with 1.0 /l mg BAL.

Maximum number of multiple shoots were found at 2.5 mg/l BAP both at 15 DAI and 30 DAI and these were 3.53 per explant and 4.20 per explant respectively. Tadhani *et al* (2006) found that, the maximum number of shoots was obtained on MS medium supplemented with 0.6 mg / 1 BAP. Mausumi Debnath (2008) reviewed that 2 mg/l along with 1.13 mg/L IAA was found to be most effective in initiating multiple shoot proliferation at the rate of 39 micro shoots per nodal explant after 30 day of culture. In respect of average length of multiple shoot, the highest responses were observed at 1.5 and 2.5 mg/l 5 and the length were 6.05 cm, 5.92 cm and 4.25 cm respectively. Highest number of node per shoot was found at 1.0 and 1.5 mg/l BAP at 15 DAT and

at 1.0, 1.5, 2.5 mg/l at 30 DAI. Lowest influence was obtained from the concentration of 2.0 mg/l at 15 DAI and 0.5 mg/l at 30 DAI. In respect of average number of leaf / shoot, highest value was noticed at 1.0 mg/l (5.60) and lowest value from 2.0 mg/l (4.00) at 15 DAI.

Table 1 Effect of different concentrations of BAP in MS medium on shoot proliferation and callus induction from shoot tip and nodal explans

Growth regulator BAP (mg/l)	Average number of multiple shoot/ Plant		Average length of multiple shoot (cm)		Average number of node per shoot		Average number of leaf /shoot		Intensity of callus induction
	X	Y	X	Y	X	Y	X	Y	
MS + 0.5	3.00 b	3.00 c	4.24 c	2.40 b	2.40 b	7.40 b	4.80 c	14.80 b	+
MS + 1.0	3.00 b	3.00 c	5.60 b	2.80 a	2.80 a	8.00 a	5.60 a	16.00 a	++
MS + 1.5	3.00 b	3.60 b	5.92 a	2.60 a	2.60 a	7.90 a	5.20 b	15.80 a	++
MS + 2.0	3.00 b	3.30 b	5.56 b	2.00 c	2.00 c	7.40 b	4.00 d	14.80 b	+++
MS + 2.5	3.53 a	4.20 c	6.05 a	2.40 b	2.40 b	7.90 a	4.80 c	15.80 a	+++
±SE(%) →	0.101	0.093	0.053	0.073	0.058	0.058	0.058	0.263	

Mean followed by similar letters in each column are not significantly different at 5 % level according to Duncans Multiple Range Test.
+= Poor, ++=Moderate, +++=High, X=15 Days After Inoculation, Y=30 Days After Inoculation

Callus was formed at the basal portion of the explants. Higher intensity of callus was found at 2.0 and 2.5 mg/L BA and lowest at 0.5 mg/L. Kabir (2005) found that MS medium supplemented with BAP (7.0 mg/L) was best medium for callus induction.

Fig. 1 Effect of BAP 2.0 mg/L on multiple shoot proliferation in MS medium

4.1.2. Effect of NAA on shoot proliferation

Four concentrations of NAA (0.5, 01.0, 1.5, 2.0 and 2.5 mg/l) were used in the recent experiment. In respect of average length of shoot, the most effective response was found with the concentration of 2.0 mg/l at 15 DAI and 2.5 mg/l at 30 DAI and the length were 5.26 cm and 8.40 cm respectively (Table-2) .The worst results were found from the concentration of 0.5 mg/L of NAA and those were 3.44 cm and 6.50 cm respectively. But Maharile *et al.* (2003) observed that MS medium containing 5 mg/L IBA+ 2 mg/L NAA resulted in the highest number of healthy shoots (46.9 shoots per culture). With respect to average number of node formed per shoot, the maximum number of node proliferated with 2.5 mg/L at 15 DAI and that was 5.00 nodes per shoot and minimum from 1.0 mg/L and that was 2.00 nodes per shoot. 1.5, 2, 2.5, mg/L NAA showed maximum number of nodes per shoot at 30 DAI and minimum with 0.5 and 1.0 mg/L. MS medium supplemented with concentration of 2.5 mg/L NAA showed highest value and lowest with 1.0 mg/L at 15 DAI. Maximum number of leaf per shoot was proliferated with 1.5, 2.0 and 2.5 mg/L and minimum with 0.5 mg/L at 30 DAI.

Table 2 Effect of different concentrations of NAA in MS medium on shoot proliferation from shoot tip and nodal explant

Growth regulator NAA(mg/l)	Average length of shoot (cm)		Average number of node /shoot		Average number of leaf /shoot		Rate of root induction (%)	Growth vigor
	X	Y	X	Y	X	Y		
MS + 0.5	4.24 c	6.50 e	3.00 bc	5.00 b	6.00 c	8.00 c	80.0	+++
MS + 1.0	3.44 d	7.44 c	2.00 c	5.20 b	4.00 d	10.40 b	80.0	+++
MS + 1.5	4.30 b	8.02 b	3.00 bc	7.00 a	6.00 c	14.00 a	80.0	++
MS + 2.0	5.26 a	7.02 d	4.00 ab	6.80 a	8.00 b	13.60 a	80.0	++
MS + 2.5	3.22 e	8.40 a	5.00 a	7.20 a	10.00 a	14.40 a	90.0	+++
±SE(%) →	**0.009**	**0.037**	**0.516**	**0.368**	**0.577**	**0.368**		

Mean followed by similar letters in each column are not significantly different at 5 % level according to Duncans Multiple Range Test.
++=Moderate vigor, +++=High vigor, X=15 Days After Inoculation, Y=30 Days After Inoculation

Subsequent root induction was observed with same concentrations of NAA in MS media. Satisfactory root induction was enhanced by all the concentrations specially with 2.5 mg/L NAA and the rate of root formation was 90%. Slavova, Nenkova and Ivanova (2003) reported that, the highest rooting percentage (99%) was obtained with 0.3 mg/l benzymidazol + 0.1 mg /l NAA. 0.5, 1.0, 1.5 and 2.5 mg/L showed statistically similar result which was 80% rooting 15 DAI that was the lowest value. Maximum number of root also found when NAA was incorporated in MS medium. Islam (2004) reviewed that, among the four media the highest number of root per shoot was obtained in MS medium supplemented with 1.0mg/l NAA followed by 1.5 and 0.5 mg/l NAA respectively. Bespalhok *et al.* (1992) said that decreasing the MS salt level in rooting medium half increased the number of root/shoot and at this level 1.0 and 10 μM NAA had a beneficial effect on root induction. Swanson *et al* (1992) said that supplementation of the shoot medium with NAA (1.0 mg/l) induced shoot culture to grow roots thereby differentiating into rooted shoot culture.

Fig. 2 Effect of NAA 2.5 mg / L on shoot proliferation in MS medium

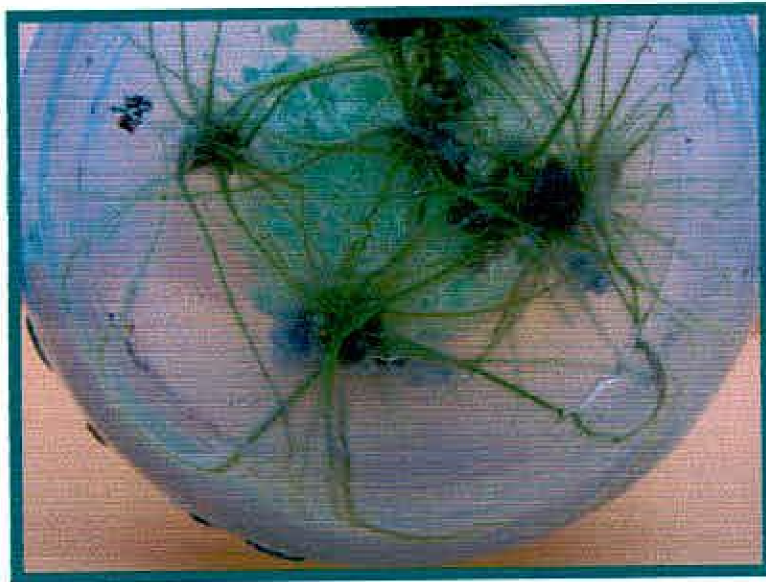

Fig. 3 Effect of NAA 2.5 mg / L on root induction in MS medium
(View from the bottom side)

4.1.3. Effect of Kn on shoot proliferation

The experiment was conducted to select the best concentration of Kn among various concentrations (0.5, 1.0, 1.5, 2.0, 2.5mg/l) on the basis of various parameters. Here shoot tips and nodes were used as explants. Related Data were presented in Table 3. Longest shoot was formed in response to Kn of 0.5 mg/l(4.94 cm) at 15 DAI and Kn 2.0 mg/l (6.96 cm) at 30 DAI. Shortest shoot was formed with Kn 2.5 mg/l at 15DAI and with Kn 1.5 mg/l at 30 DAI. Considering average number of node per shoot, highest response was observed with the treatment Kn 0.5 mg/l at 15 DAI and with Kn 2.5 mg/l at 30 DAI.

Table 3 Effect of different concentrations of Kn in MS media on shoot proliferation from shoot tip and nodal explant

Growth regulator Kn(mg/l)	Average length of shoot (cm)		Average number of node /shoot		Average number of leaf /shoot		Growth Vigor
	X	Y	X	Y	X	Y	
MS+0.5	4.94a	6.38b	3.40a	7.40b	6.80a	14.8b	+
MS+1.0	4.26c	5.94c	2.40b	5.20c	4.80b	10.4c	++
MS+1.5	3.84d	5.60e	2.30b	4.60d	4.60c	9.20d	+
MS+2.0	4.40b	6.96a	2.40b	5.20c	4.80b	10.40c	++
MS+2.5	3.60e	5.72d	2.00c	8.00a	4.00d	16.00a	++
±SE(%) →	**0.037**	**0.026**	**0.058**	**0.058**	**0.058**	**0.263**	

Mean followed by similar letters in each column are not significantly different at 5 % level according to Duncans Multiple Range Test.
+ =poor vigor, ++=Moderate vigor, X=15 Days After Inoculation, Y=30 Days After Inoculation

Mximum number of leaf was produced in response to the Kn 0.5 mg/l at 15 DAI and Kn 2.5 mg/l at 30 DAI. In case of leaf number per shoot, the poorest result was noticed with Kn 0.5 mg/l and Kn 1.5 mg/L at 15 DAI and 30 DAI respectively. Moderate vigor was maintained in the plantlets supplemented with Kn 1.0 mg/l, 2.0 mg/l and Kn 2.5 mg/L (Table 3).

4.1.4. Effect of ADS on shoot proliferation

MS medium supplemented with ADS at different concentrations (0.5, 1.0, 1.5, 2.0, 2.5 mg/L) had a significant influence on average length of shoot (Table-4). Highest length of shoot was found with ADS 2.0 mg/L (5 cm) at 15 DAI and 1.5 mg/l (6.62cm) at 30 DAI. Lowest length was found from the concentration of 2.0 and 2.5 mg/L at 15 DAI and 2.5 mg/l at 30 DAI.

Table 4 Effect of different concentrations of ADS in MS media on shoot proliferation from shoot tip and nodal explant

Hormone supplement ADs(mg/l)	Length of shoot (cm)		No. of node /shoot		No. of leaf /shoot		Rate of root induction (%)	Vigor
	15 DAT	30 DAT	15 DAT	30DAT	15DAT	30 DAT		
MS+0.5	3.0bc	5.40d	2.0	3.0ab	4.0	6.0b	50a	+
MS+1.0	2.0c	5.56c	2.0	2.0b	4.0	4.0c	40b	++
MS+1.5	4.0b	6.62a	2.0	3.4ab	4.0	6.8ab	30c	+
MS+2.0	5.0a	6.08b	2.0	4.0a	4.0	8.0a	40b	++
MS+2.5	2.5c	4.26e	2.0	4.0a	4.0	8.0a	50a	++
±SE(%)→	**0.396**	**0.045**	**0.000**	**0.448**	**0.577**	**0.547**	**0.577**	

Mean followed by similar letters in each column are not significantly different at 5 % level according to Duncans Multiple Range Test.
+ =poor vigor, ++=Moderate vigor, X=15 Days After Inoculation, Y=30 Days After Inoculation

MS media supplement with 0.5, 1.0, 1.5, 2.0 and 2.5 mg/l ADS did not show any significant difference in node and leaf formation at 15 DAI. But at 30 DAI, node and leaf formation was significantly influenced by ADS with the same concentrations. Maximum number of nodes per shoot was found with ADS 2.0 and 2.5 mg/l and lowest number of nodes per shoot was found with ADS 1 mg/l at 30 DAI. Maximum number of leaf per shoot was found with ADS 2.0 and 2.5 mg/l and minimum number of leaf per shoot was found with ADS 1.0 mg/l at 30 DAI (Table 4). But according to Aparajita *et al.* (2007), higher proliferation of shoots and multiplication were obtained on Murashige and Skoog basal medium (MS) supplemented with 1.0 mg/l indoleacetic acid (IAA) plus 10.0 mg/l kinetin (Kn) and 30.0 mg/l adenine sulphate

(ADS). Maximumroot induction (%) was observed with ADS 0.5mg/l at 15 DAI. Moderately vigorous plant growth was noticed with ADS 1.0, 2.0, 2.5 mg/l.

4.2. Study of BAP, NAA, Kn, ADS in White's media

4.2.1. Effect of BAP on shoot proliferation

White's media supplemented with different concentrations (0.5, 1.0, 1.5, 2.0 and 2.5 mg/l) of BAP did not show impressive influence on shoot length at 15 DAI but at 30 DAI, it showed marked differences and the best result was achieved with BAP 0.5 mg/l (2.5cm) and; worst with BAP 2.0 mg/L (1.86 cm) at 15 DAI and BAP 0.5mg/l at 30 DAI. Maximum number of node was proliferated with 1.0 mg/l but there was no significant difference in leaf formation at 15 DAI. Maximum number of leaf per shoot was produced with BAP 0.5 mg/L. Growth was poor with all concentrations of BAP. White's media supplemented with the concentration 0.5 mg/L BAP showed best performance both on node (3.0 node per shoot) and leaf (6.0 leaf per shoot). But M.B. Ahmed (2007) reported that, induction of multiple shoots from nodal segments was the highest in MS medium supplemented with 1.5 mg/l G BA + 0.5 mg/L Kn. All the plantlets evolved with these combination, showed poor vigor (+) (Table-5).

Table 5 Effect of different concentrations of BAP in Whites's medium on shoot proliferation from shoot tip and nodal explants

Growth regulator BAP(mg/l)	Average length of multiple shoot (cm)		Average number of node per shoot		Average number of leaf per shoot		Growth vigor
	X	Y	X	Y	X	Y	
WH+0.5	2.00a	2.50a	1.00b	3.00a	2.0a	6.00a	+
WH+1.0	1.80a	2.46b	1.20a	1.83b	2.4a	3.66b	+
WH+1.5	1.72a	2.22c	1.00b	2.00b	2.0a	4.00b	+
WH+2.0	1.36a	1.86e	1.00b	2.00b	2.0a	4.00b	+
WH+2.5	1.38a	1.88d	1.00b	2.00b	2.0a	4.00b	+
±SE(%) →	**0.258**	**0.006**	**0.026**	**0.258**	**0.259**	**0.516**	

Mean followed by similar letters in each column are not significantly different at 5 % level according to Duncans Multiple Range Test.
+ =poor vigor, X=15 Days After Inoculation, Y=30 Days After Inoculation

4.2.2. Effect of NAA on shoot proliferation and subsequent rooting

White's media supplemented with different concentrations (0.5, 1.0, 1.5, 2.0 and 2.5 mg/l) of NAA had their effect on shoot proliferetion and subsequent root formation. Highest result was found in average length of shoots with 0.5 and 1 mg/L of NAA on white's media where the lengths were 2.00 cm and 2.04 cm at 15 DAI and at 2.0 mg/l at 30 DAI. Lowest response was showed with various concentrations viz. 1.5, 2.0, 2.5 mg/L at 30 DAI. The shoot proliferation was occurred using shoot tip and nodal explant. But Smith, Nazeem, Thomas, Keshava chandra and Giriga (2005) said that , direct plantlet regeneration was induced from leaf explants on modified MS medium supplemented with 1 mg / L BA and 0.1 mg / L. In respect of node formation, highest value was observed with 2.0 mg/L both at 15 and 30 DAI (2.00 and 3.00 nodes per shoot).

Table 6 Effect of different concentrations of NAA in White's medium on shoot proliferation and rooting from shoot tip and nodal explant

Growth regulator NAA (mg/l)	Average length of shoot (cm)		Average number of node per shoot		Average number of leaf per shoot		Rate of root induction (%)	Growth vigor
	X	Y	X	Y	X	Y		
WH+ 0.5	2.00a	3.24b	1.60b	2.00b	3.20b	4.00b	75	+
WH+ 1.0	2.04a	2.20c	1.60b	1.80c	3.20b	3.60b	80	++
WH+ 1.5	1.46b	2.30c	1.50b	2.00b	3.00c	4.00b	80	++
WH+ 2.0	1.44b	4.58a	2.00a	3.00a	4.00a	6.00a	80	+++
WH+ 2.5	1.42b	2.00d	1.60b	2.00b	3.20b	4.00b	90	+++
±SE(%) →	0.026	0.045	0.058	0.058	0.058	0.368		

Mean followed by similar letters in each column are not significantly different at 5 % level according to Duncans Multiple Range Test.
+= poor vigor, ++=Moderate vigor, +++=High vigor, X=15 Days After Inoculation, Y=30 Days After Inoculation

Sritongkum (1995) reviewed that the optimum condition for root formation was occurred both an MS medium supplemented with 0.01 mg/L NAA. Considering number of leaf per shoot, best influence was noticed at 2.0 mg/L of NAA both at 15 and 30 DAI and those were 4.0 leaves per shoot and 6.0 leave per shoot respectively as presented in (Table 6).

4.2.3. Effect of Kn on shoot proliferation and subsequent root induction

Different concentrations (0.5, 1.0, 1.5, 2.0 and 2.5 mg/L) of Kn were incorporated on white's medium to observe the effect of shooting and subsequent rooting as presented in Table-7. Longest shoot was induced with 1.5 mg/L Kn both at 15 and 30 DAI from shoot tip and nodal explants where the lengths were 3.0 cm and 6.9 cm respectively. Shortest shoot was evolved with Kn 2.0 mg/L both at 15 DAI and 30 DAI and those were 1.5cm and 2.5cm respectively. Kabir (2005) used cytokinins viz. BAP and Kn for shoot proliferation in four concentrations (0.0, 1.0, 2.0, 3.0 mg/l). With respect to node and leaf formed from single shoot, the highest value was found with 2.5 mg/L

both at 15 and 30 DAI. Maximum number of nodes and leaves per shoot were found from 2.5 mg/l both at 15 DAI and 30DAI and minimum at 0.5 and 2.0 mg/l at 15 DAI (table-7). Shoot vigor was poor.

Table 7 Effect of different concentrations of Kn in White's medium on shoot proliferation and rooting from shoot tip and nodal explant

Growth regulator Kn (mg/l)	Average Length of shoot (cm)		Average Number of node per shoot		Average Number of leaf per shoot		Rate of root induction (%)	Shoot vigor
	X	Y	X	Y	X	Y		
WH+ 0.5	2.5ab	3.15c	2.20b	3.00b	4.4b	6.0b	70	+
WH+ 1.0	2.0bc	3.00c	1.00d	2.20b	2.0c	4.4b	70	+
WH+ 1.5	3.0a	6.90a	2.00c	3.00b	4.0b	6.0b	70	+
WH+ 2.0	1.5c	2.50d	1.00d	2.50b	2.0c	5.0b	40	+
WH+ 2.5	2.5ab	4.70b	3.00a	5.00a	6.0a	10.a	70	+
±SE (%) →	0.262	0.052	0.045	0.263	0.368	0.517		

Mean followed by similar letters in each column are not significantly different at 5 % level according to Duncans Multiple Range Test.
+ = poor vigor, X=15 Days After Inoculation, Y=30 Days After Inoculation

According to Constantinoviei and Cachita –Cosma (1997), shoot apices performed better as explants than nodal stem sections. Highest number of shoot tip and nodal explants produced root on 0.5, 1.0, 1.5 and 2.5 mg/l Kn (kinetin) (70%) and which was statistically different from 2.0 mg/l (40%) Kn on full strength of white's medium. But Constantinovici *et al.* (1997) said that, roots emerged from the internodes on either of the rooting media (growth regulator free or containing kinetin).

Fig. 4 Effect of Kn 2.0 mg/L on shoot proliferation in White's medium

Fig. 5 Effect of Kn 1.5 mg/L on shoot proliferation in White's medium

4.2.4. Effect of ADS on shoot proliferation

There was no significant difference among the length of shoots with different concentrations of ADS (0.5, 1.0, 1.54, 2.0, 2.5 mg/L) at 15 DAI as presented on Table 8. Longest shoot (2.5 cm) was emerged with ADS 0.5 mg/l and shortest (1.86 cm) with ADS 2.0 mg/l.

White's medium supplemented with ADS 1.0 mg/l had produced maximum number of node per explant (1.2) at 15 DAI. Minimum number of nodes found with ADS 0.5, 1.5, 2, 2.5 mg/l at 15 DAI and those were statistically similar (1.0).Maximum number of node per explant was found with ADS 0.5 mg/l at 30DAI. Minimum number of nodes per

Table 8 Effect of different concentrations of ADS in Whites's media on shoot proliferation from shoot tip and nodal explant

Growth regulator BAP(mg/l)	Average Length of shoot (cm)		Average Number of node /shoot		Average Number of leaf /shoot		Growth vigor
	X	Y	X	Y	X	Y	
WH+0.5	2.00a	2.50a	1.00b	3.00a	2.0a	6.00a	+
WH+1.0	1.80a	2.46b	1.20a	1.83b	2.4a	3.66b	+
WH+1.5	1.72a	2.22c	1.00b	2.00b	2.0a	4.00b	+
WH+2.0	1.36a	1.86e	1.00b	2.00b	2.0a	4.00b	+
WH+2.5	1.38a	1.88d	1.00b	2.00b	2.0a	4.00b	+
±SE (%) →	**0.258**	**0.006**	**0.026**	**0.258**	**0.259**	**0.516**	

Mean followed by similar letters in each column are not significantly different at 5 % level according to Duncans Multiple Range Test.
+ = poor vigor, X=15 Days After Inoculation, Y=30 Days After Inoculation

explant was found with ADS 1.0, 1.5, 2.0, 2.5 mg/L at 30 DAI. All the concentrations showed the similar effect on leaf per explant (2.0) at 15 DAI. Highest number of leaf per shoot (6) was found at ADS 0.5 mg/L at 30 DAI. Other concentrations of ADS did not show statistically significant difference on number of leaf per shoot. White's media with different concentrations (1.0, 1.5, 2.0, 2.5 mg/L) showed poor performance (Table 8).

4.3. Effect of 2,4-D on callus induction in MS and White's media

Effect of 2,4-D in response to MS and White's medium on callus induction from leaf and internode was observed and the results were presented in Table-9. Kuntal Das *et al.* (2005) said that , the initiation of callus was achieved after supplementing the various concentrations and combinations of auxins; i.e. IAA, IBA, NAA and 2,4- D, each at 1 mg/L.

Higher amount of callus were obtained from leaf segments than nodal and internodal segments. MS media supplemented with 2,4-D 2mg/l produced moderate density of callus from internodal explant. Same concentration in MS medium produced poor quality callus from leaf explant. White's media supplemented with similar concentration of 2,4-D (2mg/L) from both leaf and nodal explant developed poor intensity of callus. Uddin *et al* (2006) studied that leaf, nodal and internodal segments of the selected herb as explants were cultured on MS medium containing 2,4-D at 2,3,4 and 5 mg/l for callus induction. They also stated, the highest amount of callus was found in MS medium with 3.0 gm/l 2,4-D and MS medium with 5.0 mg/l 2,4-D gave poorest callus. Kuntal Das *et al.*(2006) observed that, extensive callus initiation was observed with 2,4-D at 1.0 mg/litre + kinetin at 0.2 mg/litre. In the recent experiment, 2,4-d at 2 mg/l was used for initiating callus. MS and Whites media supplemented with 2,4-D 2 mg/l developed whitish brown and brown colored callus respectively from internodal explant and pale green colored callus produced from leaf explant. Compact callus was developed with all the treatments in MS and White's media with the same concentration.

Table 9. Callus induction using 2,4-D in different media

Growth regulator 2,4-D(mg/L)	Explant used	Color	Texture	Intensity
MS+2.0	Internode	Whitish brown	Compact	++
MS+2.0	Leaf	Pale green	Compact	+
WH+2.0	Internode	Brown	Compact	+
WH+2.0	Leaf	Pale green	Compact	+

+ =Poor, ++=Moderate

4.4. Effect of IBA on root induction in different media

Easy root formation is very important for the convenient establishment of *in vitro* regenerated plantlets. For successful micro propagation, healthy and strong root system is required. In the present experiment, explants were transplanted in different media viz. half MS, half While's, half LS and half Phytamax (PM) media. All the media were in solid, semi-solid and liquids condition .IBA 1 mg/L was used in the recent study.

Table 10 Formation of roots in response to IBA (1mg/L) in different media at 15 DAI

		Number of shoot root/Shoot	Average length of root (cm)	% Root
Media condition	Solid	3.00 a	2.35 a	79.50 c
	Semi-solid	2.50 a	1.85 b	73.25 b
	Liquid	2.68 a	1.58 c	55.00 a
	±SE(%) →	**0.210**	**0.087**	**0.236**
Media	MS	4.00 a	3.00 a	98.67 a
	White	3.07 a	2.57 b	98.33 a
	LS	1.33 b	1.43 c	36.67 c
	PM	1.91 b	0.70 d	43.33 b
	±SE(%) →	**0.242**	**0.101**	**0.272**

Mean followed by similar letters in each column are not significantly different at 5 % level according to Duncans Multiple Range Test.

Maximum number of root per shoot was induced in MS medium supplemented with IBA mg/L. There was no statically significant different in number of root per shoot using the MS medium in solid, semi-solid and liquid condition. That is solid, semi-solid and liquid MS media were combinedly proved as the best media for producing inducing maximum number of root per explant. LS, PM and White's media had minimum effect on number root production per shoot and any statically significant difference was not found considering the sane parameter with same treatment. Condition of media had no effect on number of root production per shoot.

In respect of length of root, highest length was noticed in MS medium at solid condition supplemented with IBA 1mg/l. Ferreira *et.al.* (1988) found that shoot

elongation and further rooting of isolated shoot was better on LS medium devoid of growth regulators. Variation in rooting capacity, plant vigor morphological characters was found amongst regenerated plants. With the same treatment liquid medium gave lowest length. Rate of root induction (%)was best in both MS and White's media in liquid condition and was worst in LS medium with liquid condition with the same treatment (Table-10). Aparajita Mitra and amita pal (2007) observed that profuse roots were induced from 90% of the regenerated shoots within 4 weeks of induction on $^{1}/_{2}$ strength MS solid medium supplemented 1.0 mg/L IAA. Morini, *et.at* (2003) used IBA at 0.5 mg/L and 0.1 mg/L to evaluate shoot rooting response.

At a glance, solid MS medium showed the best performance in respect of root production. Ms medium showed the better response than PM., LS and White's media .It may be due to presence of higher amount of sucrose, macro and micro nutrients in MS medium than that of other media.

Fig. 6 Effect of 2,4-D 2.0 mg/L on callus induction in MS medium

Fig. 7 Effect of IBA 1.0 mg/L on root induction in liquid MS medium

4.5. Combined effect of organic supplements and PGRs on shoot proliferation in different media

In this study different concentrations of different PGRs (BAP, Kn, NAA, IBA, IAA) and various concentrations (2 mg/L, 4mg /L) of organic supplements (Orange juice and Tomato juice) were added in LS and PM media. Significant affect was found in all parameters studied (Table 12). Highest length of shoot was found with PM media supplemented with IBA 0.5 mg/L +IAA 0.5mg/L +Tomato juice 2mg/L both at 15DAI and 30 DAI and those were 3.5 cm and 9.0 cm respectively. Lowes length was found with the combination LS+BAP 1 mg/L +NAA 0.5 mg/L +Orange juice 2 mg/L. Highest length of shoot was found with PM media supplemented with IBA 0.5 Best response was found with PM media supplemented with IBA 0.5 mg/L, IAA 0.5 mg/L and Tomato Juice 2mg/L from shoot tips and nodal explants considering all the parameters except growth vigor. Sivaram *et al.* (2003) said that, shoot apex ,nodal and leaf explants of *S. rebaudiana* Bertoni can regenerate shoot when cultured on MS medium supplemented with BA 8.87 μM and IAA 5.71μM .

Table 11 Combined effect of PGRs and organic supplements on shoot proliferation in different media

Media plus PGR and organic supplement	Average length of shoot (cm)		Average number of node /shoot		Growth vigor
	X	Y	X	Y	
LS+0.5BAP+0.5Kn + 2 mg/L O.J.	3.00b	4.00b	3.00b	4.00b	+
LS+ 1BAP+0.1Kn+4 mg/L T.J.	2.70c	3.50c	3.00b	4.20b	++
LS+ 1BAP+0.5 NAA+ 2 mg/L O.J.	2.50d	3.00d	2.00c	3.00c	++
PM + 2 mg/L T.J.	2.80bc	3.00d	3.00b	3.00c	+
PM +2Kn+ 2 mg/L O.J.	3.00ab	3.30d	3.00b	3.00c	+
PM + 0.5 IBA+0.5 IAA+ 2 mg/L T.J.	3.50a	9.00a	4.00a	9.00a	+
±SE (%) →	**0.058**	**0.337**	**0.242**	**0.337**	

Mean followed by similar letters in each column are not significantly different at 5 % level according to Duncans Multiple Range Test.
+ = poor vigor, ++ = Moderate vigor, O.J.-Orange Juice ,T.J.-Tomato Juice, X=15 Days After Inoculation, Y=30 Days After Inoculation

Moderate growth vigor of plantlets was noticed through visual observation with the combined treatment of LS+BAP 1 mg/L+ Kn 0.1 mg/L+ Tomato juice 4 mg/L and LS + BAP 1 mg/L + NAA 0.5 mg/L + Orange Juice 2 mg/L. Other combination showed

poor vigor. Shoot tip and internode were used as explants as used in other studies, in the research .Huda *et at.* (2007) reported that leaf segments, internode and nodal segments of stevia were cultured on MS medium supplemented with varying concentration (1, 3, 5 and 7 mg/L) of growth regulators (2,4-D BAP and NAA) along with coconut water to observe morphogenic responses.

Fig. 8 Shoot proliferation LS +1 mg/L BAP+0.1 mg/L Kn+4mg/L Tomato juice

4.6. Interaction effect of different PGRs, their concentrations and media on shoot proliferation

PGR, their concentrations and associating media had a great effect on shoot proliferation of *Stevia rebudiana* as showed in Table 12.

4.6.1. Effect of different concentrations

In the study five different concentrations (0.5,1.0, 1.5, 2.0, 2.5 mg/L) were used. In shoot length the progressive response was not found with the increasing concentrations as observed in number of leaf produced per shoot. Treatment with 2.5 mg/L gave maximum number of leaf (5.10) and minimum with 0.5 mg/L (3.80) considering shoot length, highest value was found at 1.5 mg/L (2.19 cm) and lowest at 0.5 mg/L (1.33cm). In respect to number of node and leaf per shoot, superior result was observed at 2.5 mg/L of concentration and those were 2.55 and 5.10 respectively. Treatment 1.0 mg/L showed lowest result in number of node per shoot which was 1.78. Increase in concentration with different hormones (BAP, NAA, Kn, ADS) on different hormone showed a progressive result in the perimeter of number of leaf per shoot (Table12). 15 were days required for the recent investigation.

4.6.2. Effect of different PGRs

In the recent investigation BAP, NAA, kn and ADS hormone were used to observe the shoot proliferation on MS and White's medium with different concentrations (0.5, 1.0, 1.5, 2.0, 2.5 mg/L) as presented in Table 12. Highest length was found with ADS (2.20 cm) and lowest with BAP (1.42 cm) but haider (2006) reported that MS basal medium supplemented with 13.5 micro M benzyladenine (BA) + 8.0 micro M NAA showed best performance.

Table 12 Effect of different PGRs, their concentrations and media on shoot proliferation

Concentration	Length of Shoot (cm)	Number of Node/shoot	Number of leaf/shoot	Day required
0.5	1.33 b	1.90 cd	3.80 c	15
1.0	2.03 d	1.78 d	3.58 c	15
1.5	2.19 a	2.06 bc	4.13 b	15
2.0	1.73 c	2.19 b	4.38 b	15
2.5	2.12 ab	2.55 a	5.10 a	15
±SE →	**0.035**	**0.074**	**0.113**	
PGR				
BAP	1.42 d	3.20 a	6.41 a	15
NAA	2.09 b	1.57 c	3.14 c	15
Kn	1.83 c	2.44 b	4.90 b	15
ADS	2.20 a	1.17 d	2.34 d	15
±SE →	**0.031**	**0.067**	**0.101**	
Media				
MS	2.25	3.20	6.40	15
White's	1.52	1.00	2.00	15

Mean followed by similar letters in each column are not significantly different at 5 % level according to Duncans Multiple Range Test.

Maximum number of nodes (3.20) per shoot was produced with BAP and lowest (1.17) with ADS. It happened due the stimulation of BAP to produce multiple shoots Smitha *et al.* (2005) also reviewed that, multiple shoot were induced from shoot buds on modified MS medium supplemented with 1.0 mg / L benzyladenine (BA) Maximum number of nodes (6.41) produced with BAP and lowest (2.34) with ADS which was similar to the node formation from shoot tips and nodal explants .

4.6.3.Effect of different media

Shoot tip nodal explants were cultured in MS and White's media. Longest shoot (2025cm) was found in MS medium and shortest shoot (1.52cm) in White's medium. Highest number of node (3.20) and leaf (6.4) were found in MS medium and minimum number of node (1.0) and leaf (2.0) were found in White's medium.

According to the above discussion, it can be said that longest shoot was found with ADS 1.5 mg/L poor response was found with BAP 0.5 mg/l incorporated in White's medium. (Table 12). In respect of number of node per shoot, MS medium with BAP 2.5 mg/l was proved as the best medium and poor response with White's medium with ADS 1.0 mg/L. In respect of number of leaf per shoot, MS medium supplemented with BAP 2.5 mg/L gave the best response and white's medium supplemented with ADS 0.5 mg/L and ADS 1.0 mg/L gave poor response.

MS medium was proved as the medium according to all the performances. It may be due to the presence of Myo-Inositol which is absent in White's medium. The amount of macro and micronutrients and sugar in MS medium is also higher than that present in White's medium. These factors may be responsible for better shoot proliferation in MS medium. BAP was proved as the best PGR considering all parameters. It may be due to effective physiological influence found with BAP than other PGRs.2.5 mg/l was proved as the most suitable concentration .It may be due to the optimum level of concentration which enhance the growth and multiplication of plantlets.

4.7. Synergistic effect of BAP and Kn

The synergistic effect of BAP and Kn with MS and white's media was observed and presented in the Table-13 .In the recent study, two treatments were used on the both media. All the parameters studied significantly influenced by BAP 2mg/L combined with Kn 1 mg/L. Highest length was found from shoot tip and nodal explants with BAP 2mg/l + Kn 1 mg/L with MS medium (2.5cm) and lowest length with BAP 2mg/L + Kn 2 mg/L on White's medium at 10 DAI. Bondarev *et al.* (2003) observed that, adding of 0.1 mg/L BA or 6-benzy/aminoparine, to gather with alpha-naphthalene acetic acid resulted in on 1.5 fold increase in number of shoot.

Table 13 Synergistic effect of BAP and Kn

Growth regulator BAP + Kn (mg/l)	Length of Shoot (cm)		Number of Node/shoot		Number of leaf/shoot		Growth vigor
	X	Y	X	Y	X	Y	
2+1(MS)	3.5a	5.0a	2.0a	7.8a	4.0b	15.6a	+
2+2(MS)	2.7c	5.5a	4.0b	7.0b	8.0a	15.0a	+
2+1(WH)	3.0b	5.5a	2.0b	3.0d	4.0b	6.0c	+
2+2(WH)	1.5d	2.5b	2.0b	4.0c	4.0b	8.0b	+
±SE (%) →	**0.058**	**0.293**	**0.293**	**0.410**	**0.577**	**0.501**	

Mean followed by similar letters in each column are not significantly different at 5 % level according to Duncans Multiple Range Test.
+ =poor vigor, X=15 Days After Inoculation, Y=30 Days After Inoculation

There was no statistically significant different among the results with MS+2mg/L BAP+1 mg/L Kn, MS+2 mg/l BAP +1 mg/L Kn and WH+ 2mg/L BAP+1mg/L Kn in shoot length at 30 DAT. Maximum number of node per explant was produced with MS+2mg/l BAP+ 2mg/L Kn (4.0) at 15 DAI. Kornikova and Kallashnikova (1996) said that use of MS medium without growth regulator gave poor result those obtained with growth regulator (BA + NAA and Kn + NAA) using segment with two auxiliary bud. There was no statistically significant influence node production with other concentration at 15 DAI. Maximum number of node (7.8) and leaf (15.6) per shoot was observed with 2 mg/L BAP+1mg/L Kn on MS medium at 30 DAI and lowest result was found with 2mg/L BAP+1mg/L Kn on while's medium at 30 DAI. All the plantlets produced in response to combined concentration of hormones were not vigorous.

4.8. Transfer of plantlets to soil and post transplantation observation

In the recent study various combinations of soil containing cowdung, poultry litter and chemical fertilizer (P Urea , MP, TSP) were used to select the best medium.

In chemical fertilizer, the proportion of NPK was 40:20:30 kg/ha. Combination 2 gave highest survival rate (80%) and lowest with combination 3 and 4. Goenadi (1985)

reported that, plant growth and leaf number / plant was increased by FYM and liquid fertilizer. Average length of plant (32.1 cm) was maximum with combination 2. Shortest length (22.2 cm) was found with combination 1 and 4. Highest number of leaf per plant was produced with the combination 2 (70) and lowest with combination 1 and 2. Highly vigorous plants were grown with combination 2. Combination 2 contains cowdung. That means cowdung improved the soil fertility markedly than other supplement. Angkapradipta, Warsito and Faturachim (1986) said that, seedlings in pots received a basal dressing of 200g cattle manure and urea at 0-2.0 g, triple super phosphate at 0-0.25 g and potassium chloride at 0-0.20g/pot.Cattle manure markedly improved soil fertility. Increasing rates of N increased plant N content, and reduced soil PH, K, Ca and Mg. Poor quality plants grown with combination 1.

Table 14 Growth observation of plantlets transplanted in pots

Soil combination	Number of plantlet transplanted	Plantlet survived	Survival rate (%)	Average length (cm)	Number of leaf per plant	Average time required (Days)	Growth vigor
Combination 1	10	7a	70b	22.2c	48c	60	+
Combination 2	10	8a	80a	32.1a	70a	60	+++
Combination 3	10	5b	50c	30.3b	68b	60	++
Combination 4	10	5b	50c	22.2c	44c	60	++
±SE (%) →		**0.577**	**0.577**	**0.577.**	**0.577**		

Mean followed by similar letters in each column are not significantly different at 5 % level according to Duncans Multiple Range Test.
+ =poor vigor, ++=Moderate vigor, +++=High vigor

Average time required was 60 days for the study. Buana and goenadi (1985) said that, plant height and leaf number were measured weekly in *Stevia rebaudiana* cuttings fertilized with various levels of NPK, farm yard manure or a 'Super Mineral' fertilizer. Lee *et al.* (1980) reported that N, P_2O_5 and k_20 at rates of 0,10 and 15 kg/10 a each, applied in various combinations did not affect *Stevia rebaudiana* plant height, number of branches or number of internodes but the dry leaf yield was best when 10kg of each nutrient was applied. Kornienko *et al.* (1995) observed that PABK (P-

aminobenzoic acid) at 0.005% plus 80 kg N + 80 kg P2O5 + 80 kg K2O/ha was the best treatment producing 53.9 and 13.79 t/ha fresh and dry biomass respectively. Kuntal Das *et al.* (2006) studied the effect of combined applications of nitrogen (N), phosphorous (P) and potassium (K) fertilizers on their availability in soils in relation to their contents in the Stevia plant. The results show that the amount of available N, P and K in soil has been found to be increased initially up to 45 days and thereafter, the amount of the same content decreased with the progress of plant growth up to 60 days irrespective of treatments. However, the magnitudes of such increases in N, P and K contents both in soils and plants have also been enhanced with the simultaneous application of N, P and K (40:20:30) kg/ha over that of their corresponding individual applications.

Fig. 9 Plants grown in soil supplemented with poultry litter

Fig. 10 Plants grown in soil supplemented with cowdung

Fig. 11 Plants grown in soil without supplementation

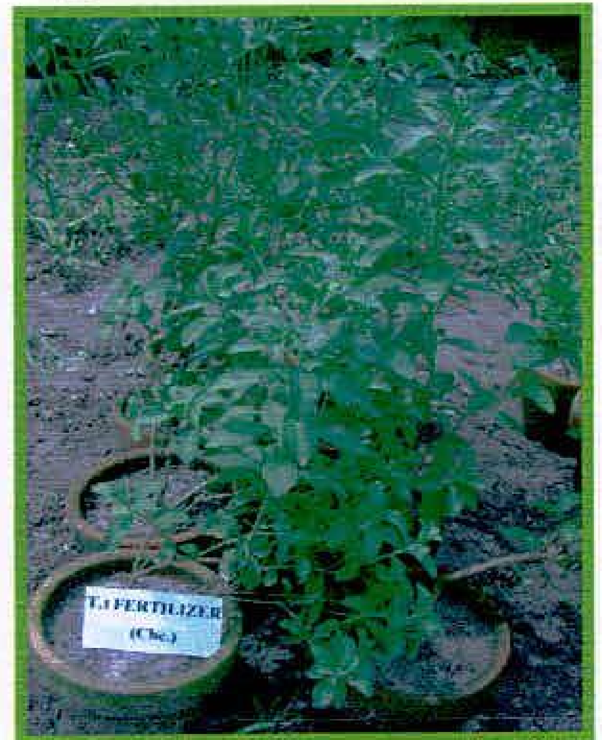

Fig. 12 Plants grown in soil supplemented with chemical fertilizers (NPK)

Chapter 5

Summary and Conclusion

CHAPTER 5

SUMMARY AND CONCLUSIONS

The experiment was conducted at the plant Tissue Culture Center Laboratory of Proshika Plant Tissue Culture Trust (PPTCCT), koitta, Manikgong, Blangladesh. Leaf and nodal segment were used as explant for *in vitro* plant regeneration. For comparative study, MS, PM, LS and White's media were used supplemented with different growth regulators viz. BAP, NAA, Kn and ADS at various concentration (0.5,1.0, 1.5, 2.0, and 2.5 mg/L). For callus induction 2, 4-D 2mg/L was incorporated in MS, ½ Whit's, ½ LS and ½ PM media. All the media were in solid, semi-solid and liquid condition. Two types of organic supplement viz. Orange Juice and Tomato juice were used with various concentration of BAP, Kn, NAA and IAA in both LS and MS media. To study the synergic effect of BAP and Kn, these PGRs were incorporated in Ms and White's medium, For this investigation and Kn, 1 and 2 mg/L along with BAP mg/L were used. Four soil combinations containing cowdung, poultry litter and chemical fertilizer (NPK) were used.

The following conclusions could be drawn from the present investigations

Best media for shoot proliferation using single PGR

Highest length was found with MS medium supplemented with ADS 1.5 mg/L. Maximum number of node for med per shoot was found in MS medium with BAP 2.5 mg/L. Maximum number of node per shoot was obtained with BAP 2.5 mg/L.

At a glance, MS medium with 2.5 mg/L was proved as the best medium considering all the parameters for shoot proliferation.

Best medium for shoot proliferation using organic supplements and PGRs

Best response was found with PM media supplemented with IBA 0.5 mg/L + IAA 0.5 mg/L + 2 mg/L tomato juice evaluating all the parameter used except growth vigor. Moderate growth vigor of plantlets was noticed through visual observation with treatment of LS+ mg/L BAP and LS + mg/L BAP + 0.5 mg/L NAA + 2 mg/L orange juice.

Best media for shoot proliferation in response to PGR synergism

Highest length was found from shoot tip and nodal explants with BAP 2 mg/L + Kn 1 mg/L in MS medium (2.25cm). Maximum number of node (2.02) per shoot was produced with BAP 2 mg/L + kn 2 mg/L in MS medium at 15 DAI. Maximum number of node (7.8) and leaf (15.6) per shoot was observed with BAP 2 mg/L+ Kn 1 mg/L in MS medium at 30 DAI. All the plantlets produced in response to combined concentration of PGR were not vigorous.

At a glance, MS medium supplemented with BAP 2 mg/L + Kn 1 mg/L showed best influence considering average shoot length, number of node per shoot and number of leaf per shoot.

Best media for root induction using single PGR solid, semi-solid and liquid

MS media were combined proved as the best for producing maximum number root induced per shoot supplemented with IBA 1 mg/L.

Best media for callus induction

Highest intensity of callus were obtained from leaf segments than inter nodal segments. MS media with 2,4-D 2 mg/L had given best response when incorporated in MS medium in respect of intercity of callus induced.

Best soil combination

Average length of plant (32.1 cm) and number of leaf (70) per plant were maximum with combination 2. Combination 2 gave highest survival rate (80%). So combination 2 was proved as the best treatment.

Chapter 6

References

REFERENCES

Angkapradipta, P., Tuti Warsito and Katiman, M. 1986.Manuring of *Stevia rebaudiana* Bertoni M. on andosol. *Menara-Perkebunan.* **54**: 6, 131-137; 4.

Aparajita, M. and Amita, P.2007.*In-vitro* regeneration of Stevia rebaudiana (Bert) from the nodal explants. *Journal-of-Plant-Biochemistry-and-Biotechnology.* **16(1)**: 59-62.

Barciszewski, J., Siboska, G.E., Pedersen, B.O., Clark, B.F.C. and Rattan, S.I.S. (1996) .Evidence for the presence of kinetin in DNA and cell extracts. *FEBS Letters*: 393, 197-200.

Bespalhok-Filho-JC and Hattori-K.1997. Embryogenic callus formation and histological studies from *Stevia rebaudiana* (Bert.) Bertoni floret explants. *Revista-Brasileira-de-Fisiologia-Vegetal.***9**: 3, 185-188; 25.

Bespalhok, Filho,J.C., Vieira, L.G.E. and Hashimoto,J.M. 1992. Factors influencing the *in vitro* micropropagation of axillary shoots of Stevia rebaudiana (Bert) Bertonil. *Revista-Brasileira-de-Fisiologia-Vegetal.* **4**: 1, 59-61; 2 pl.; 15.

Bondarev,N. L., Novosm, A. M. and Kornienko, A.V.1998. Effects of exogenous growth regulators on callogenesis and growth of cultured cells of *Stevia rebaudiana* Bertoni. *Rus. J. Plant Physiol* .**45(6)**: 770-774.trasplanted from *Fiziol . Ras* .45(6) 888-892.

Bondarev, N. Reshetnyak,O. and Nosov, A. 2003. Effects of nutrient medium composition on development of *Stevia rebaudiana* shoots cultivated in the roller bioreactor and their production of steviol glycosides. *Plant-Science.* **165(4)**: 845-850.

Buana, L. and Goenadi,D.H.1985. A study of growth patterns of stevia cuttings. *Menara-Perkebunan.***53**: 4, 124-133; 6 ref.

Carnerio, J.W.P., Muniz, A.S. and Guedes ,T.A.1997.Grenhouse bedding plant production of *Stevia rebaudiana* (Bert.)Bertony.*Can .J. Plant sci.***77**: 473-474.

Carvalho,M.A.M., Zaidan,L.B.P. and Carvalho,M.A.M. 1995. Propagation of *Stevia rebaudiana* from stem cuttings. *Pesquisa-Agropecuaria-Brasileira.* **30**: 2, 201-206; 26 ref.

Chalapathi, M.V. and Thimmegowda, S. 1997. *Stevia rebaudiana* using large scale bioreactor.Natural non-calorie sweetener stevia (*Stevia rebaudiana* Bertoni). *Plant Cell Rep.* **13**: 3-4, 180-183.

Chalapathi, Thimmegowda, Devakumar, Chandraprakash and Rao.1999. Vegetative propagation of stevia (*Stevia rebaudiana* Bertoni) under field conditions. *Crop-Research-Hisar.***18(2)**: 319-320.

Constantinovici, D. and Cachita and Cosma,D.1997. Aspects of in vitro multiplication in *Stevia rebaudiana* Bert. *Cercetari-Agronomice-in-Moldova.* **30**: 4, 80-86; 6.

Debnath, M. 2008.Clonal propagation and antimicrobial activity of an endemic medicinal plant *Stevia rebaudiana. Journal of Medicinal Plants Research.* **Vol. 2(2)**, pp. 045-051.

Dubey, H., Kumar, M.and Haider .2006. *In vitro* morphogenesis of Stevia: the naturally occurring sweet plant - a fast and efficient method to propagate Stevia by tissue culture. *Journal-of-Research*, **18(1)**: 1-9.

Ferri, L.A.F., Alves-do-Prado,W., Yamada,S.S., Gazola, S., Batista, M.R. and Bazotte ,R.B. 2006.Investigation of the antihypertensive effect of oral crude stevioside in patients with mild essential hypertension. Phytotherapy-Research..**20(9)**: 732-736.

Ferreira, C. M. and Handro,W.1988.Production, maintenance and plant regeneration from cell suspension cultures of *Stevia rebaudiana* (Bert.) Bertoni. *Plant Cell Reports,* **Volume** 7,Page123-126.

Geuns, J.M.C. 2003 . Stevioside. Phytochemistry. 2003. **64(5):** 913-921.

Goenadi,D.H.1985. Effect of farmyard manure, NPK, and liquid organic fertilizers on *Stevia rebaudiana. Menara-Perkebunan*. **53**: 2, 29-34; 7.

Handro,W. Hell,K.G. and Kerbauy,G.B.1977. Tissue culture of *Stevia rebaudiana*, a sweetening plant. *Planta-Medica*. **32**: 2, 115-117; 3 pl.; 12.

Hawke, J. 2002. STEVIA Natural Sweetener For Everyone. NEXUS Magazine.

Hopkins W. G. (1999). Introduction to Plant Physiology. John Wiley and Sons. ISBN 0-471-19281-3

Islam and M . T. 2004. In vitro propagation of stevia (*Stevia rebaudiana* Bert.).Under Graduate thesis .Khulna University , Khulna ,Bangladesh.

Kabir, A. H. M. S .2005. *In vitro* propagation and utilization of stevia (*Stevia rebaudiana Bert.*). MS thesis. Bangladesh Agricultural University, Mymensing, Bangladesh.

Kinghorn, A. D. 1992.Food ingredient safety review:Stevia rebaudiana leaves.Herb Research Foundation , Boulder.

Kornienko,A.V., Nikul ,I.M., Udovidchenko,L.P., Bezler,N.V., Kurakov,V.I., Zhuzhalova,T.P. and Znamenskaya,V.V. 1995. Stevia cultivation. *Sakharnaya-Svekla*. **No. 10**, 22-24.

Kornilova,O.V. and Kalashnikova,E.A. 1996. Clonal micro-propagation of stevia (Stevia rebaudiana Bertoni). *Sakharnaya-Svekla.* **No. 11**, 20-22.

Kuntal D., Raman D. and Shivananda, T. N.. 2006. Effect of N, P and K fertilizers on their availability in soil in relation to the Stevia plant (*Stevia rebaudiana* Bert.). *Archives of Agronomy and soil.* Volume 52, Issue 6, pages 679 - 685

Kuntal, D. and Raman, D. 2005. Stevia - a sugar substitute for diabetic patients. *Crop-Research-Hisar*. **30(2)**: 305-308.

Kuntal, D., Raman,D. and Shivananda. 2006. Effect of major nutrients on biomass production and stevioside content of *Stevia rebaudiana* - a pot culture study. *Biomed.* **1(1)**: 22-25.

Latha S. and Usha M. 2003 *In Vitro* culture studies on *Stevia rebaudiana* In Vitro Cellular and Developmental Biology. *PlantArticle*: pp. 520–523.

Lester, T. 1999.*Stevia rebaudiana.* (Sweet Honey Leaf). Australian New Crops Newsletter. Issue no.11.

Lee, J.I., Kang, K.H., Park, H.W., Ham, Y.S. and Park, C.H.1980. Studies on the new sweetening source plant, *Stevia rebaudiana* in Korea. II. Effects of fertilizer rates and planting density on dry leaf yields and various agronomic characteristics of *Stevia rebaudiana.* Research-Reports-of-the-Office-of-Rural-Development,-Crop,-Suwon. **22**: 138-144; 6.

Liu, J. and Li, S.F.Y. 1995. Separation and Cercetari-Agronomic-in Moldova. *Planta Medica,***18 (9)**: 1703-1719. **54 (2)**: 157-160.

Maharik, N.T. and Gengaihi, S.E.2003. Micropropagation of *Stevia rebaudiana* Bertoni. *Egyptian-Journal-of-Horticulture.* **30(1/2)**: 125-134.

Morini, S., Fiaschi, G. Andolfi, L. and Macchia, M. 2003. In vitro propagation of *Stevia rebaudiana* Bertoni: results with different genotypes. *Agricoltura-Mediterranea.* **133(2)**: 117-123.

Mousumi Debnath. 2008.Clonal propagation and antimicrobial activity of an endemic medicinal plant *Stevia rebaudiana.Joumal* of Medicinal Plants Research Vol. 2(2), pp. 045-051.

Murashige T. and Skoog F.1962. A reviced medium for rapid growth and biossays with tobacco tissue cultures . *Physiol. Plant* **15**:473-497.

Oddone, B.1997. How to grow stevia . Technical manual . Guarani Botanicals, Pawtuket CT.

Pude, R .2005. 300 times sweeter than sugar and without calories. Stevia cultivation is viable. *Gemuse-Munchen*. **41(10)**: 27-28.

Slavova,Y. Nenkova, D. and Ivanova, I .2003. Study on the influence of the substance of benzymidazol upon *Stevia rebaudiana* Bertoni cultivated *in vitro*. *Bulgarian-Journal-of-Agricultural-Science*. **9(2)**: 225-228.

Smitha, P.S., Nazeem, P.A., Thomas, J., Keshavachandran, R and Girija, D. 2005. Micropropagation for mass multiplication of the important medicinal sweet herb - *Stevia rebaudiana*. *Journal-of-Medicinal-and-Aromatic-Plant-Sciences*. **27(2)**: 247-252.

Sritongkum, P. 1995 .Micropropagation of *Stevia rebaudiana* Bertoni.M. Sc. Thesis. Chulanlongkorn University.

Sumathi, M; Samuel, S.D. and Parthiban, S. 2005. Stevia - a way to sweeten diabetics life. *Indian-Coffee*. **69(10)**: 12-15.

Swanson, S.M. Mahady, G.B. and Beecher,C.W.W. 1992. Stevioside biosynthesis by callus, root, shoot and rooted-shoot cultures *in vitro*. *Plant-Cell,-Tissue-and-Organ-Culture*. **28**: 2, 151-157; 21 ref.

Tamura.1984. *Comparison of* Stevia plants grown from seeds, cuttings and stem-tip cultures for growth and sweet diterpene glucosides. *Plant Cell Reports*, **3(5)**: p. 180-182.

Toyoda, K. and Matsui, H. 1997. Assessment of the carcinogenicity of stevioside in Bangladesh. *Afr. J. Biotechnol*. **5 (13)**: 1238-1240. F344 rats. Food 13.

Tsai, F.Y., Lin, C.C. and Kao, C.H.1997 . A comparative study of the effects of abscisic acid and methyl jasmonate on seedling growth of rice Authors. *Plant Growth Regulation.* Volume **21**, pp. 37-42(6).

Uddin, M.S., Chowdhury, M.S.H., Khan, M.M.M.H., Uddin, M.B. and Ahmed, R., Baten, M.A. 2006. *In vitro* propagation of *Stevia rebaudiana* Bert in Bangladesh. *African-Journal-of-Biotechnology.* **5(13)**: 1238-1240.

Yamazaki, T. and Flores, H.E. 1991. Examination of steviol glucosides production by hairy root and shoot cultures of *Stevia rebaudiana.* Biol. Plant. **39**: 520-523.

Yang, Y.W.and Chang, W.C. 1979. *In vitro* plant regeneration from leaf explants of *Stevia rebaudiana* Bertoni. *Zeitschrift-fur-Pflanzenphysiologie.* **93**: 4, 337-343; 2 pl.; 9.

Yoshida, S. 1986. Studies on the production of 16 sweet substances in *Stevia rebaudiana:. Crop Sci.* **55 (2)**: 189-195.

Tamura, Y., Nakamura, S., Fukui, H.and Tabata, M. 1984. Comparison of Stevia plants grown from seeds, cuttings and stem-tip cultures for growth and sweet diterpene glucosides. *Plant Cell Reports.* Volume **3**, Pages 180-182.

Zbughin, G. Sturzu, T.M. and Constantinovici,-D. 2002. Some aspects about "*in vitro*" multiplication process of *Stevia rebaudiana* (Bertoni) Hemsl. (Asteraceae).*Analele-Stiintifice-ale-Universitatii-'-Al-I-Cuza'-din-Iasi-Serie-Noua-Sectiunea-II-Biologie-Vegetala.* **48**: 43-52.

Appendices

APPENDICES

Appendix-1

Preparation of MS (Murashige and Skoog, 1962) medium

Sl. No.		
Major Salts (10X)		**g/l**
1.	Potassium nitrate (KNO_3)	19.00
02.	Ammonium nitrate (NH_4NO_3)	16.50
3.	Magnesium Sulphate ($MgSO_4.7H_2O$)	3.70
4.	Calcium Choloride ($CaCl_2.2H_2O$)	4.40
5.	Potassium phosphate (KH_2PO_4)	1.70
Minor Salts (100X)		**mg/l**
1.	Potassium iodide (KI)	83
2.	Boric Acid (H_3BO_3)	620
3.	Manganese Sulphate ($MnSO_4,4H_2O$)	2230
4.	Zinc Sulphate ($ZnSO_4$, $7H_2O$)	860
5.	Sodium Molybdate (Na_2MoO_4, $2H_2O$)	25
6.	Copper Sulphate ($CuSO_4$, $5H_2O$)	2.5
7.	Cobalt Chloride ($CoC1_2$, $6H_2O$)	2.5
Iron EDTA solution (100 X)		**g/l**
1.	Ferrous sulphate ($FeSO_4$ $7H_2o$)	2.78
2.	Sodium – EDTA (Na_2EDTA, $2H_2O$)	3.73
Organics (100X)		**mg/l**
1.	Myo-Inositol	10000
2.	Nicotinic acid	50
3.	Pyridoxine HCL	50
4.	Thiamine HCL	10
5.	Glycine	200
Other additives		
1.	Sucrose	0-30 g/l
2.	Agar	6-8 g/l

Preparation of White's medium

Appendix-2

Sl.No.		
Major Salts (10X)		**mg/l**
1.	Potassium nitrate (KNO_3)	80
2.	Potassium Choloride (KCl)	65
3.	Magnesium Sulphate ($MgSO_4.7H_2O$)	737
4.	Calcium Nitrate $Ca(NO_3)_2.4H_2O$	288
5.	Sodium Sulphate (Na_2SO_4)	460
6.	Sodium Biphosphate (Na_2HPO_4	19
Minor Salts (100X)		**mg/l**
1.	Potassium iodide (KI)	0.75
2.	Boric Acid (H_3BO_3)	1.5
3.	Manganese sulphate ($MnSO_4.4H_2O$)	6.65
4.	Zinc sulphate ($ZnSO_4. 7H_2O$)	2.67
5.	Molybdenum Oxide (MoO_3)	0.0001
6.	Copper sulphate ($CuSO_4. 5H_2O$)	0.001
Iron EDTA solutioon (100 X)		**mg/l**
1.	Ferreus sulphate ($FeSO_4, 7H_2o$)	27.8
2.	Sodium – EDTA ($Na_2EDTA, 2H_2O$)	33.6
Organics (100X)		**mg/l**
1.	Nicotinic acid	0.5
2.	Pyridoxine HCL	0.1
3.	Thiamine HCL	0.1
4.	Glycine	3.0
Other additives		
1.	Sucrose	2000

Preparation of Phytamax medium

Appendix-3

Sl.No.		
Major Salts (10X)		**mg/l**
1.	Potassium nitrate (KNO_3)	950
2.	Calcium Choloride ($CaCl_2$)	166
3.	Magnesium Sulphate ($MgSO_4.7H_2O$)	90.45
4.	Ammonium nitrate (NH_4NO_3)	825
5.	Potassium Biphosphate ($KHPO_4$)	85
Minor Salts (100X)		**mg/l**
1.	Sodium molybadate (Na_2MoO_4, $2H_2O$)	0.125
2.	Boric Acid (H_3BO_3)	3.1
3.	Manganese sulphate ($MnSO_4.4H_2O$)	8.45
4.	Zinc sulphate ($ZnSO_4$. $7H_2O$)	5.3
5.	Cobalt chloride ($CoCl_2$, $6H_2O$)	0.0125
6.	Copper sulphate ($CuSO_4$. $5H_2O$)	0.0125
7.	Potassium iodide (KI)	0.415
Iron EDTA solution (100 X)		**mg/l**
1.	Ferreus sulphate ($FeSO_4$, $7H_2o$)	27.85
2.	Sodium – EDTA (Na_2EDTA, $2H_2O$)	37.24
Organics (100X)		**mg/l**
1.	Nicotinic acid	0.50
2.	Pyridoxine HCL	0.50
3.	Thiamine HCL	1.00
4.	Myo-Inositol	100
5.	**Pepton**	2000
Other additives		
1.	Sucrose	200000

Linsmiaier and Skoog medium Appendix-4

Sl.No.		
Major Salts (10X)		**mg/l**
1.	Potassium nitrate (KNO_3)	1900
2.	Calcium Choloride ($CaCl_2$)	332.2
3.	Magnesium Sulphate ($MgSO_4.7H_2O$)	
4.	Ammonium nitrate (NH_4NO_3)	1650
5.	Potassium Phosphate (K_3PO_4)	170
Minor Salts (100X)		**mg/l**
1.	Molybdic acid	2.5
2.	Boric Acid (H_3BO_3)	6.2
3.	Manganese sulphate ($MnSO_4.4H_2O$)	16.9
4.	Zinc sulphate ($ZnSO_4$. $7H_2O$)	8.6
5.	Cobalt chloride ($CoC1_2$. $6H_2O$)	0.025
6.	Copper sulphate ($CuSO_4$. $5H_2O$)	0.025
7.	Potassium iodide (KI)	0.83
8.	Magnesium sulphate ($MgSO_4$)	180.7
Iron EDTA solution (100 X)		**mg/l**
1.	Ferreus sulphate ($FeSO_4$. $7H_2o$)	27.8
2.	Sodium – EDTA (Na_2EDTA, $2H_2O$)	37.25
Organics (100X)		**mg/l**
1.	Thiamine HCL	0.4
2.	Myo-Inositol	100
Other additives		
1.	Sucrose	200000

Sher-e-Bangla Agricultural University
Library
Accession No ...27216...
Sign: ...R... Date: 04-12-13

95 (...) Hort.
13/09/09