CALLUS INDUCTION AND PLANT REGENERATION OF STEVIA (*Stevia rebaudiana* B.)

BY

JANNATUL FERDOUS

REGISTRATION NO. 08-02964

A Thesis
Submitted to the Faculty of Agriculture,
Sher-e-Bangla Agricultural University, Dhaka,
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE
IN
BIOTECHNOLOGY

SEMESTER: JANUARY-JUNE, 2015

Approved by:

Dr. Md. Ekramul Hoque
Professor
Department of Biotechnology
SAU, Dhaka-1207
Supervisor

Md. Abdul Halim
Assistant Professor
Department of Biotechnology
SAU, Dhaka-1207
Co-supervisor

Dr. Md. Ekramul Hoque
Professor
Department of Biotechnology
SAU, Dhaka-1207
Chairman
Examination Committee

Prof. Dr. Md. Ekramul Hoque
Department of Biotechnology
Sher-e Bangla Agricultural University
Dhaka-1207, Bangladesh
Mob: +88 001712836595

CERTIFICATE

This is to certify that thesis entitled, **"CALLUS INDUCTION AND PLANT REGENERATION OF STEVIA (*Stevia rebaudiana* B.)"** submitted to the Faculty of **AGRICULTURE,** Sher-e-Bangla Agricultural University, Dhaka, in partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE** in **BIOTECHNOLOGY**, embodies the result of a piece of bona fide research work carried out by **JANNATUL FERDOUS,** Registration No. **08-02964** under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.

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

Dated: June, 2015
Place: Dhaka, Bangladesh

.....
(Prof. Dr. Md. Ekramul Hoque)
Supervisor

DEDICATED
TO
MY BELOVED PARENTS

ABBREVIATIONS AND ACRONYMS

Agril.	:	Agriculture
Biol.	:	Biological
Biot.	:	Biotechnology
cm	:	Centimeter
Conc.	:	Concentration
DAI	:	Days After Inoculation
WAI	:	Week After Inoculation
et al.	:	And others (at elli)
i.e.	:	id test(That is)
FAO	:	Food and Agricultural Organization
g/L	:	Gram per litre
BAP	:	6- Benzyl Amino Purine
BA	:	Benzyladenine
KIN	:	Kinetine
IAA	:	Indole acetic acid
IBA	:	Indole butyric acid
NAA	:	*a*- Napthalene acetic acid
2, 4-D	:	2,4- Dichlorophenoxy acetic acid
Int.	:	International
J.	:	Journal
Mol.	:	Molecular
mg/L	:	Milligram per litre
µM	:	Micromole
MS	:	Murashige and Skoog
PGRs	:	Plant Growth Regulators
Res.	:	Research
Sci.	:	Science
TDZ	:	Thidiazuron
CV	:	Co-efficient of Variation
°C	:	Degree Celsius
etc.	:	Etcetera
w/v	:	Weight by volume

ACKNOWLEDGEMENTS

First of all I would like to thanks to Almighty Allah, the great, gracious, merciful and supreme ruler of the universe who has given me the ability for successful completion of the research, preparation of manuscript, its submission in time as a partial requirement for the degree of MS(Master of Science) in Biotechnology.

After that I would like to express my sincere and deepest sense of gratitude, heartfelt thanks and respect to my research supervisor, ***Prof. Dr. Md. Ekramul Hoque*** *, Department of Biotechnology, Sher-e-Bangla Agricultural University, Dhaka-1207, for selecting me to work on Stevia rebaudiana B. and also for his scholastic guidance, uncompromising principles, valuable advises, constant inspiration, constant suggestions and constructive criticism during this investigation and in the preparation of this manuscript.*

I would like to offer my deep sense of respect, sincere gratitude, and immense compulsion to my respected co-supervisor ***Md. Abdul Halim****, Assistant Professor, Department of Biotechnology, Sher-e-Bangla Agricultural University, Dhaka-1207, for extending his guidance, lab support, continuous counseling and constant encouragement during the research work and preparation of the manuscript of the thesis.*

I express sincere appreciation and boundless gratitude to ***Homayra Huq****, Associate Professor, Chairman, Department of Biotechnology, Sher-e-Bangla Agricultural University, Dhaka-1207, for her valuable teaching, sympathetic co-operation, guidance and inspirations throughout the course of this study and research work.*

I express my respect to honorable course teacher, ***Mohammad Nazrul Islam****, Assistant Professor, Department of Biotechnology, Sher-e-Bangla Agricultural University, Dhaka-1207, for his valuable teaching and advice.*

I am proud to express my deepest gratitude to the Ministry of Science and Technology for NST fellowship and financial supporting.

I would like to convey a special word of thanks to ***Prof. Dr. Md. Razzab Ali****, Department of Entomology, Sher-e-Bangla Agricultural University, Dhaka-1207, for his co-operation with data analysis.*

I wish to extend warm thanks to all the staff including tissue culture laboratory of Department of Biotechnology, Sher-e-Bangla Agricultural University for their friewndly relationship, friendly gesture and for their help directly or indirectly regarding my work.

I express my gratitude and heartfelt thanks to the alumni of Department of Biotechnology for their suggestion and all of my friends who have been there for me through the challenging times. I will always appreciate the support and love that they gave me.

Finally I found no words to thank to my beloved family specially my father and mother. I only would like to take this opportunity to express my profound gratitude to my parents, and family for their unquantifiable love, continuous support, supreme sacrifice and unshakable faith in me that has always helped me to proceed further.

And after all I sincerely wish that this thesis work could help researchers who are involved in tissue culture all over the world and can contribute to the scientific knowledge of plant regeneration ,which are widely used in tissue culture.

The Author

CALLUS INDUCTION AND PLANT REGENERATION OF STEVIA

(*Stevia rebaudiana* B.)

BY

Jannatul Ferdous

ABSTRACT

The present research work was carried out in Biotechnology laboratory of the Department of Biotechnology, Sher-e-Bangla Agricultural University, Sher-e-Bangla Nagar, Dhaka-1207 from the period of January 2014 to December 2014 to evaluate the effects of different plant growth regulators in *in vitro* on shoot proliferation and root formation and finally to develop a viable micropropagation protocol in Stevia (*Stevia rebaudiana*) using shoot tip and nodal segments as explants. Explants was inoculated in MS (Murashige and Skoog) media supplemented with the Benzyladenine (BA) and Kinetine (KIN) for direct shoot formation. Combination of BA and Indolebutyricacid (IBA) as well as combination of KIN and IBA were used in MS medium for indirect shoot formation via callus induction. The highest percent of shoot induction (83.42%) obtained on 2.0 mg/L BA and the minimum days to shoot induction (10.6) were also obtained on 2.0 mg/L BA. The highest 10.8 shoots and 17.2 leaves per explants also observed in BA 2.0mg/L. But maximum average length of shoots per explants (3.94 cm) were reported from media containing 1mg/L KIN. Highest callus induction potentiality (83.58%) was observed in 2 mg/L BA+1.5 mg/L IBA .The minimum days to callus induction (13.4) were recorded in 1.0 mg/L KIN+1.5 mg/L IBA but the minimum days to shoot induction (9.6) from callus were recorded in 2.0 mg/L BA+1.5 mg/L IBA. Highest percent of root induction (78.18%) with 7.2 roots per explants and minimum 12.6 days to root induction were noticed on 1.0 mg/L IBA. The combined effect on percent of root induction was highest (76.92%) in the treatment of 1.0 mg/L KIN + 0.5 mg/L IBA. The same treatment showed minimum days (13.4) to root induction and maximum number of roots per explants. In acclimatization 90%, 80% and 80% survival rate were recorded in growth cabinet, shade house and field condition respectively. *In vitro* regeneration technique was found to be very effective and promising method in the proliferation of *Stevia* and this method can be used for large scale production of disease free and quality planting material.

TABLE CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF APPENDICES

Chapter I
Introduction

CHAPTER I

INTRODUCTION

Stevia rebaudiana Bertoni is the botanical name of stevia. This plant is a natural sweetener and famously known as "Sweet Weed", "Sweet Leaf', "Sweet Herbs" and "Honey Leaf (Yadav, 2016). Now a days, *Stevia* is becoming very popular one among various medicinal plants for the treatment of different diseases in our country as well as all over the world. *Stevia* is a herbaceous perennial plant native to subtropical and tropical rainforest areas of South America (Brazil, Venezuela, Colombia and Paraguay). Stevia belonging to family Asteraceae which are small, semi-bushy, perennial shrub with sessile, oppositely arranged lanceolate to oblanceolate leaves serrated above the middle. The mature plant grows up to 65 cm (26 inches) to as tall as 180 cm (72 inches) when cultivated or growing naturally in fertile soil. It is a short day plant and flowering from January to March in the southern hemisphere. It prefers a sandy soil, requiring a warm sunny position. The suitable natural climate is semi humid subtropical with temperature extremes from 21 to 43°C and average 24°C (Huxley, 1992). Stevia grows in areas with up to 1375mm of rain a year. The plants are not very frost resistant.There are more than 180 species of the *Stevia rebaudiana* that gives the sweet essence (Soejarto *et al*., 1982).

This natural sweetener Stevia is a good source of proteins, carbohydrates and fiber. It has both economical and medicinal importance. Stevia plant used as a traditional herbal remedies for diabetes among many diseases around the world. The leaves have been known to contain 100 useful alkaloids among other pharmacologically active compounds which can be used for the treatment of diabetes. It has a special importance to diabetic persons and diet conscious people i.e. its products can be used as a substitute of artificial sweeteners for diabetic patients. Stevia leaf extract exhibits a high degree of antioxidant activity and has been reported to inhibit human cancer cell growth. It has healing effect on blemishes, wound cuts and scratches. Its other medicinal uses include regulating blood sugar, preventing hypertension and tooth decay as well as treatment of skin disorders. Stevia is helpful in weight and blood pressure management. Stevioside, the bio-active compound in its leaves, tastes about 300 times sweeter than sucrose and used as a sweetening agent in industrial sector and is commercially important. Stevia is regenerated as a valuable natural sweetening agent because of its relatively good taste and chemical stability (Yamazaki, 1991). It is gaining a lot of importance for the production of diterpene glycosides which are nutritive, non-toxic, high-potency sweeteners, and may be used as a substitute of sucrose as well as other synthetic

sweeteners. Due to the non-caloric and sweetening properties stevioside has gathered attention with the rise in demand for low carbohydrate, and low sugar food alternatives (Kalpana *et al.*, 2009). Products can be added to tea and coffee, cooked or baked goods, processed foods and beverages, fruit juices, tobacco products, pastries, chewing gum and sherbets. Stevia had been used for removing the bitter taste of medicinal plants. In Japan alone, 50 tones of stevioside are used annually with sales valued in order of 220 million Canadian dollars (Brandle and Rosa, 1992). The leaves are used traditionally in various regions of the world including China, Japan, Korea, Taiwan, Thailand, Malaysia and Paraguay. Thousands of people in Bangladesh are suffering from diabetic mellitus and high blood pressure. So stevioside may play a very important role for the treatment of this people (Uddin *et al.*, 2006). On the other hand this plant has many industrial importance in Bangladesh perspective.

Stevia is an exotic plant in our country. There are basically two options for multiplication: tissue culture and stem cutting (Puri *et al.*, 2011). Conventionally, it is cultivated by seeds or stem cutting, but seed viability rate is poor. Due to poor seed viability and low germination rate, the common method of propagation by seed is restricted. Vegetative propagation by stem cuttings is also limited by the low number of individuals that can be obtained simultaneously from single plant. Medicinal and commercial value of Stevia lead to the world wide demand for large-scale production of this plants from elite germplasm. Keeping in view its utmost medicinal values in account, in vitro culture study was attempted to raise the stevia plants through effective protocol.

Plant tissue culture is a suitable approach for micro propagation and production of valuable secondary metabolites of plants. The technique of Plant Biotechnology has an important role to play in the production of agriculture, horticulture and ornamental plants and in the manipulation of plants for improved agronomic performance. Propagation through tissue culture can be suitable as an alternative method to obtain sufficient number of plants within short period of time (Ibrahim *et al.*, 2008). Micropropagation can provide genetically uniform plants in large numbers. Micropropagation of *S. rebaudiana* through in vitro techniques has been reported (Janarthanam *et al.*, 2009; Moktaduzzaman and Rahman, 2009; Kalpana *et al.*, 2009; Das *et al.*, 2011). Different explants of stevia can regenerate shoots when cultured on murashige and skoog (MS) medium supplemented with different combination of growth regulators (Ramesh *et al.*, 2006). A reproducible protocol for *in vitro* regeneration is a prerequisite for improvement of Stevia propagation. It is important to standardize the protocol of explants response for callus induction and *in vitro* regeneration of Stevia. Both

callus induction and plant regeneration from explant require the presence of appropriate combinations and concentrations of phytohormone in the culture media. Individual hormone has its own effect on regeneration. Combined effect of hormone on rooting and shooting is needed to study for better regeneration of Stevia. Therefore, the present investigation has been carried out to identify the best hormonal combination and concentration for Stevia regeneration. On the above mentioned perspective, the present study was undertaken to develop and establish a reproducible protocol for plantlet regeneration of *Stevia.*

Objectives:

The present investigation has been carried out with the following objectives:

1. Establishment of callus induction and plant regeneration protocol of *Stevia.*
2. Assessment of hormonal effect on micropropagation of *Stevia* in *in vitro.*
3. Acclimatization and cultivation of *in vitro* regenerated plantlets of *Stevia.*

Chapter II
Review of literature

CHAPTER II

REVIEW OF LITERATURE

Plant tissue culture is the newest route in the science of cell biology. Tissue culture is the process of regeneration in an artificial nutrient medium under aseptic condition. The idea of plant tissue culture originated from the cell theory that was formulated by Schwann in 1839. Development of new organized structures i.e. organs from the old one through tissue culture is done by two ways: direct and indirect. Emergence of adventitious organs directly from explants known as direct method. Indirect is the process of regeneration new organs through callus formation. Callus is an unorganized mass of cells that develops when cells are wounded and is very useful for many *in vitro* cultures. Auxins and cytokinins both aid in the formation of most callus cells (Ali *et al.*, 2007). Callus can be continuously proliferated using plant growth hormones or then directed to form organs or somatic embryos. Tissue culture is now applied in many sector of biology for rapid propagation. Like many other countries in Bangladesh different government and non-government organizations are working in this area. Many agricultural, medicinal, forest crops etc. is now under tissue culture in our country. *Stevia* is one of them. Many plant breeders of different countries as well as Bangladesh have been employing biotechnological tools for the development of *Stevia,* an important medicinal plant. But it is very limited in Bangladesh. Related works already performed by different institutes of the world have been reviewed and some of the most relevant literatures are cited here under different headings.

2.1 Explant and Growth conditions

Tiwari *et al.* (2013) studied on rapid micropropagation of *Stevia rebaudiana* Bertoni .They concluded their research by saying that plant tissue culture is an essential component of plant biotechnology which offers novel approaches to the production, propagation, conservation and manipulation of plants. The success of *in vitro* culture of *Stevia* depends mainly on the growth conditions of the source material medium.

Giridhar *et al.* (2010) stated that shoot tip when cultured in B_5 medium supplemented with BAP (4.4 μM) and NAA (0.8 μM) produce maximum number 28 shoots produce from a single explants. The number of explants increase through repetitive cycle. Elongation of microshoot is better in B_5 medium devoid of growth regulator. *In vitro* rooting of microshoot was good in MS (Murashige and Skoog)basal medium.

An experiment was conducted by Rafiq *et al.* (2007) to optimize growth medium and growth conditions for *in vitro* propagation using nodular stem sections of *Stevia rebaudiana* Bertonie.

The effects of different media containing IAA(indole acetic acids), IBA(indole butyric acids) and NAA (α-Napthalene Acetic Acid) (0.5 and 1 mg/L) in combination with BA and kinetin (1 and 2 mg/L) on one step cultures of stevia axillary buds were investigated by Taleie *et al.* (2012).

An experiment was conducted by Uddin *et al.* (2006) on *in vitro* culture of *Stevia rebaudiana* Bert. Where leaf, nodal and inter-nodal segments of the selected herb as explants were cultured on MS medium.

Karim *et al.* (2008) ran the experiment to develop and establish a reproducible protocol for plantlet regeneration in Stevia. Nodal cutting explants were cultured in MS medium supplemented with different concentrations and combinations of NAA and 6-Benzylamino purine (BAP).

A protocol for callus induction and multiplication was developed by Gupta *et al.* (2010) to produce large no. of calli in short period. Surface sterilized nodal, leaf and root explants of *Stevia rebaudiana* B. were cultured on MS medium with different concentrations of plant hormone like, IBA, kinetin, NAA, 2,4-D, and NAA in combination with 2,4-D.

Micropropagation protocols of *Stevia rebaudiana* B., an important medicinal plant of Asteraceae family have been developed by Singh and Dwivedi (2013) using nodal segments in half strength MS media.

Initiation of callus had been done by Pasha (2014) using explants from auxiliary and apical meristems of *Stevia rebaudiana.* Explants were inoculated on MS basal medium having Vitamin supplement with Auxin like 2-4, D and NAA (0.5mg/L -2.0mg/L) alone or in combination with cytokinis like BAP & KIN (0.52 mg/L -1.0 mg/L).

Micropropagation of *Stevia rebaudiana* via temporary immersion bioreactor system was successfully conducted by Noordin *et al.* (2012). Shoot tips and nodal segment were used as explants to induce multiply shoots.

Experiment of Jitendra *et al.* (2012) highlighted the development and achievements made for the micropropagation of *Stevia rebaudiana* Bertoni (an antidiabetic sweetener herb). Shootlets were regenerated from nodal explants of stem through auxiliary shoot proliferation.

Shende and Manik (2013) used young axillary buds as explants for rapid multiplication of *Stevia rebaudiana*. The explants were cultured on medium containing basal salts of Murashige and Skoogs (MS) and various concentrations of BAP for shoot induction.

MS basal medium with 1.0 mg/L of BAP was found the best medium for shoot formation, with 90% shoot formation response within 12 days of meristem inoculation, both from shoot apical and nodal meristem (Ali *et al.*, 2010).

Soliman *et al.* (2014) studied on in vitro propagation of Stevia through multiple shoot regeneration from nodal segments cultured on MS medium supplemented with various concentrations of BA, NAA, IAA and thidiazuran (TDZ).

Hossain *et al.* (2008) conducted an experiment where *in vitro* propagation of *Stevia* was attempted by multiple shoot regeneration from the explants (shoot tips and nodal segments) followed by rooting of multiplied shoots. Shoot tips showed better response for shoot proliferation than nodal segments.

The aim of Namdari *et al.* (2015) study was to develop a suitable protocol for micro propagation of *Stevia rebaudiana* Bertoni using node and axillary shoots.

Verma *et al.* (2011) established an effective protocol for propagation of *S. rebaudiana* using nodal explants from young branches and then cultured on BAP (0.5-2.0 mg/l) in combination with Kn or NAA (0.2-0.5 mg/l).

Kalpana *et al.* (2009) observed in their research that shootlets were regenerated from nodal explants of stevia through axillary shoot proliferation.

An attempt had been made to standardize a protocol for direct shoot regeneration from leaf explants of *S. rebaudiana* by Preethi *et al* (2011). Among the combination of plant growth regulators tested by them in field grown leaf explants, maximum number of shoots (10.4±0.21) was obtained on MS medium supplemented with BA (1.0 mg/L), KIN (0.5 mg/L) and IAA (0.1 mg/L), where as in *in vitro* derived leaf explants maximum number of shoots (28.7±0.84) was obtained on MS medium supplemented with BA (2.0 mg/L), Kn (0.5 mg/L) and NAA (0.1 mg/L).

In vitro regeneration protocol was standardized by Pawar *et al.* (2015) for propagation of promising newly introduced non caloric sweetener and an antidiabetic medicinal plant Stevia. Nodal segment and shoot tip was used as an explants.

A rapid and reproducible protocol for in vitro regeneration of *Sphaeranthus indicus* (Asteraceae), a medicinal herb had established by Yarra *et al.* (2010). Leaf segments isolated from mature plants were cultured on MS medium with different concentrations of BA (2.2, 4.4, 6.6 and 8.8 μM) or KIN (1.3, 2.3, 4.6 and 6.9 μM).

2.2 Sterilization of explants

Both NaOCl and $HgCl_2$ are oxidizing agents and damage the microorganism by oxidizing the enzymes (Rao, 2008). The ineffectiveness of NaOCl may be due to the reason that it is a mild sterilizing agent (Sirivastava *et al.*, 2010). $HgCl_2$ is reported a better sterilizing agent as compared to NaOCl but is more toxic and requires special handling and is difficult to dispose (Maina *et al.*, 2010).

Verma *et al.* (2011) recorded data of *Stevia* which were sterilized by using different concentration of bavistin and mercuric chloride ($HgCl_2$).They showed that the best results in sterilization of nodal explants were recorded with using Bavistin 0.2% + 8-HQC (200ppm) for 1 hour.

Jagatheeswari and Ranganathan (2012) found sterilization of explants with lower concentration of mercuric chloride treatments with lesser timings gave the best result.

Surface sterilization of *Stevia* with $HgCl_2$ for 3 minutes gave best result of disinfection with maximum survival of explants for nodal segment and shoot tip also is proved by Pawar *et al.* (2015).

2.3 Callus and shoot induction of *Stevia*

An experiment was conducted by Uddin *et al.* (2006) on in vitro culture of *Stevia*, an important non-caloric sweetening herb to explore its potentiality for micro-propagation. Leaf, nodal and inter-nodal segments of the selected herb used as explants cultured on MS medium containing 2, 3, 4 and 5 mg/L of 2,4-D for callus induction. They found that inter-nodal segments initiated callus earlier than node and leaf. MS medium with 3.0 mg/L 2,4-D produced the highest amount of callus where MS medium with 5.0 mg/L 2,4-D gave the poorest callus.

Karim *et al.* (2008) ran the experiment to develop and establish a reproducible protocol for plantlet regeneration in Stevia. The combination of NAA at 1.0 mg/L and BAP at 1.0 mg/L resulted in the highest percentage (100%) of callus initiation. The maximum shoot regeneration and development of shoot was observed at the same combination.

A protocol for callus induction and multiplication of *Stevia* was developed by Gupta *et al.* (2010) to produce large no. of calli in short period. 100% callusing was observed from leaf explants cultured on combination of NAA and 2,4-D after three weeks while with 2,4-D, only 10% callusing was observed. Calli obtained from leaf and root explants were shiny green while with nodal explants it was hard and brown.

Pasha (2014) initiated callus of *Stevia* on MS basal medium having vitamin supplement with Auxin like 2,4-D and NAA (0.5mg/lt. - 2.0mg/L) alone or in combination with cytokinin like BAP & Kinetin (0.52 mg/lt. -1.0 mg/lt.). Their finding was maximum 60-70% callusing in 10 to 15 days was initiated on 2,4-D (1.0-2.0 mg/L) and 40-50% callusing were reported in medium containing (1.0 mg/L NAA & 0.5 mg/lt. BAP) in 25-30 days. Nodular compact cells were formed in 2,4-D. The medium containing BAP alone showed shoot formation 70-80% with 40% coconut water.

The experiment carried out by Sikdar *et al.* (2012) to establish an efficient callus induction system of *Stevia* from a variety of explants as well as direct regeneration from nodes. MS medium fortified with NAA 2.0 mg/L + BA 2.0 mg/L showed the highest (93.33 ± 6.67%) callus induction by nodal explants. Though inter-nodal explants showed a moderate response (73.33 ± 6.67%) for callus induction in MS medium complemented with NAA 3.0 mg/L + BA 1.0 mg/L, nodal explants showed higher response (86.67 ± 13.33%) than inter-nodal explants in that nutritional environment. Leaf explants always showed very poor callus. They found the best direct regenerating medium as MS medium + BA 1.0 mg/L for multiple shoot proliferation.

Single use of Kn (0.5 mg/L) as compared to BAP produced best shooting response, whereas, when various combinations of cytokinins and auxins were considered BAP and IBA (0.2 mg/l) each produced maximum shoots (2.75) of *Stevia* with moderate shoot length (1.70 cm) and good number of leaves (28) found by Singh and Dwivedi (2013).

A protocol was developed Razak *et al.* (2014) for *in vitro* micropropagation of *Stevia* where maximum shoot formation (7.82 ± 0.7 shoots per explants) was observed on a Murashige and Skoog (MS) medium supplemented with 0.5 mg L^{-1} BAP and 0.25 mg L^{-1} Kn.

Hossain *et al.* (2008) conducted an experiment on *in vitro* propagation of *Stevia* and found BAP (1.0 mg/l) superior to all other hormonal treatments for shoot proliferation. Full MS was superior to half MS. Full MS supplemented with NAA (1.5 mg/l) was the best medium for rooting of microcuttings.

Maximum shoot formation was observed by Rafiq *et al.* (2007) in their experiment by supplementing 2.0 mg/L BAP in nodular stem sections of *S. rebaudiana.*

Abd Alhady (2011) conducted an experiment on the micropropagation of *Stevia rebaudiana* using MS as the medium supplemented with different concentrations of BA at concentration, 0.0, 0.1 and 0.5 mg/L individually or in combination with Kin at concentration, 0.0, 0.1 and 0.5 mg/l for culture establishment. They found MS medium supplemented with 2.0 mg/L BAP+ 0.5 mg/L kin produced the maximum number of proliferated shoots.

Taleie *et al.* (2012) reported the effects of different media containing IAA, IBA and NAA (0.5 and 1.0 mg/L) in combination with BA and kin (1.0 and 2.0 mg/L) .The results showed that plantlets with a mean shoot length of 8 cm, number of shoots (3.77), number of inter nodes (11.5), fresh weight (0.58 g), dry weight (0.06 g).

Regeneration of shoots of *Stevia* was found by Jagatheeswari and Ranganathan (2012) on when cultured on Murashige and Skoog (MS) medium supplemented with BA (8.87 µM) and IAA (5.71 µM).

Deshmukh and Ade (2012) established in vitro rapid multiplication method for *S. rebaudiana* by inoculating explants on M.S. medium, supplemented with different combination of phytohormone. The maximum number of shoots (18.3±0.8) was obtained on M.S. medium supplemented with BAP + KIN (1.5 + 0.5 mg/L). The highest rooting percentage was observed with (IAA 0.1 mg/L).

Micropropagation of *Stevia* via temporary immersion bioreactor system was successfully conducted by Noordin *et al.* (2012). It was found that shoot tips on MS medium supplemented with 1 mg/L Kinetin showed the highest shoot multiplication after 3 weeks of culture. Shoot elongation and rooting was successfully optimized in MS basal medium 2 weeks later.

Maximum number of shoots (10.4±0.21) of *Stevia* was obtained on MS medium supplemented with BA (1.0 mg/L), Kn (0.5 mg/L) and IAA (0.1 mg/L), where as in *in vitro* derived leaf explants maximum number of shoots (28.7±0.84) was obtained on MS medium supplemented with BA (2.0 mg/L), Kn (0.5 mg/L) and NAA (0.1 mg/L) among the combination of plant growth regulators tested by Preethi *et al.* (2011). The best rooting response was observed on 2.0mg/L IBA.

For initiation shoot of *Stevia* MS + 1 mg L^{-1} BAP media was found best performing media. Maximum number of shoots per plant were observed on media MS + 2 mg L^{-1} BAP for both nodal segment and shoot tip explants. MS + 2 mg L^{-1} BAP media was found most promising media. Highest *in vitro* rooting (62.3 %) was obtained within 12.3 days on MSB_5 media supplemented with 1.0 mg/L NAA. During hardening of rooted plants, 70% plantlet survival was observed on cocopeat in green house (Pawar *et al.*, 2015).

2.4 Shoot Multiplication

Chotikadachanarong and Dheeranupattana (2013) studied multiple shoot induction of *Stevia rebaudiana* Bertoni for 4 weeks .The maximum amount of multiple shoot inducted(9.31±4.17 shoots/explant) when cultured on MS media supplemented with 3 mg L^{-1} kinetin.

Abd Alhady (2011) conducted an experiment on the micropropagation of *Stevia rebaudiana* where medium supplemented with kin resulted in elongated shoots.

In the experiment of Thiyagarajan and Venkatachalam (2012) MS medium supplemented with 1.0 mg/L BAP gave the highest frequency (94.50%) of multiple shoot regeneration with maximum number of shoots (15.69 shoots/explants) of *Stevia* and *in vitro* derived nodal bud explants were cultured on MS medium fortified with 1.0 mg/l BAP for large scale plant production, in which about 123 shoots/explants were obtained after three subcultures on the same media composition.

A procedure has been outlined by Debnath (2008) for plant regeneration and antimicrobial screening of a medicinal herb, *Stevia rebaudiana Bertoni*. MS medium supplemented with 2.0 mg/L BAP and 1.13 mg/L IAA in combination were found to be most effective in inducing bud break and growth, and in initiating multiple shoot proliferation.

Das *et al.* (2011) developed a novel protocol for accelerated *in vitro* mass multiplication in *Stevia* through multiple shoot induction and found MS basal medium supplemented with sucrose (30.0 g L^{-1}), agar (7.0 g L^{-1}) and kinetin (2.0 mg L^{-1}) performed best in multiple shoot proliferation, resulting more than 11 multiple shoots from a single shoot tip explants within 35 days of culture.

The induction of multiple shoots from nodal segments was the highest in MS medium supplemented with 0.5 mg/L BAP + 2.0 mg/L Kn. For rooting different concentration of IBA were used and highest rooting was recorded on MS medium with 1.0 mg/L IBA. The rooted Plantlets were hardened initially in culture room conditions and then transferred to mist house found by Jitendra *et al.* (2012).

Inclusion of IAA into BA supplemented medium triggered a high frequency of regeneration response from leaf explants of *Stevia*. Maximum number of shoots (12 ±1.15) with highest shoot length (3.0 ±0.73) were obtained directly (without intervening callus phase) from the leaf explants using combination of BA (4.4 µM) and IAA (1.71 µM) within 3-4 week of culture. The elongated shoots were rooted on MS medium fortified with IBA (2.46 µM) (Yarra *et al.*, 2010).

Maximum shoot elongation (3.5) along with the highest number of leaves per shoot (8.7) was observed on the medium composed of MS salts and vitamins with GA_3 in the concentration of 0.5 mg/L by Verma *et al.* (2011) for establishing an effective protocol for propagation of *Stevia rebaudiana*.

MS basal medium with 1.0 mg/L of BAP was found the best medium for shoot formation, with 90 % shoot formation response within 12 days of meristem inoculation, both from shoot apical and nodal meristem. Maximum shoot multiplication response (90%) was also obtained in MS medium having 1.0 mg/L of BAP, with average of 8.6 shoots per culture vial having an average shoot length of 6.0 cm. The best *in vitro* rooting response (96%) was recorded on MS medium containing 1.0 mg/L NAA within 7.3 days of inoculation (Ali *et al.*, 2010).

Stevia explants cultured on MS medium without plant growth regulators produced the highest shoots (4.5 cm) with two shoots per explant. The best multiplication rate of shoots were found on MS medium added with 1.13 mg/L BA combined with 0.35 mg/L IAA which produced on average 4.5 shoots and 11.9 nodes per initial explants (Sumaryono & Sinta, 2011).

The maximum number of axillary shoots of *Stevia* per explant (3.24) and highest shoot length (3.12 cm) were observed with MS medium supplemented with 1.0 mg/L BA+0.05 mg/L NAA and 2.0 mg/L BA+0.05 mg/L NAA, respectively (Soliman *et al.*, 2014).

For *in vitro* regeneration of *Stevia* frequencies of root no. observed in 2.0 mg/L IBA (3.62) and frequencies of root length observed in 2.0 mg/l IBA (3.71). While at concentration lower than 2.0 mg/l treatment resulted decline in all parameters (Yadav, 2016).

2.5 Root induction of *Stevia*

Abd Alhady (2011) used MS medium supplemented with indole butyric acid (IBA) or Naphthalene acetic acid (NAA) at concentration, 0.5, 1.0 and 2.0 mg/l was used for rooting of *Stevia* separated shoots. Medium supplemented with 1.0 or 2.0 mg/l IBA produced the maximum root induction (100%). For rooting IBA showed to be more significant and effective than NAA among all concentrations.

0.5 mg/L NAA caused the maximum root formation in nodular stem sections of *S. rebaudiana* in the experiment of Rafiq *et al.* (2007).

Root length (3.62 cm) and root numbers (12) produced on MS medium supplemented with 1.0 mg/L IAA and 1.0 mg/L BA of *Stevia* on the experiment of Taleie *et al.* (2012).

MS media supplemented with 0, 0.1, 0.5 and 2 mg L^{-1} NAA used for *In vitro* root initiation of separated shoot of *Stevia*. The highest number of roots (11.18±1.34 roots/shoot) was detected on a concentration of 0.1 mg L^{-1} NAA (Chotikadachanarong and Dheeranupattana, 2013).

Half-strength MS medium augmented with 0.4 mg/l NAA noticed the highest frequency of rooting (96%). Elongated shoots transferred to a MS rooting medium supplemented with 2.0 mg/L IBA, and this gives best rooting (Debnath, 2008).

Roots induction and maximum percentage of number of roots regeneration on MS medium supplemented with 0.1 mg/L IBA was observed by Ghauri *et al.* (2013) for *in vitro* micro-propagation of exotic medicinal plant *Stevia rebaudiana* Bert.

Shoot lets were regenerated from nodal explants of stevia through axillary shoot proliferation. For rooting different concentrations of IAA, IBA and NAA were used and highest rooting percentage (63%) was recorded on MS medium with IAA (1.0 mg/l). The rooted plantlets were hardened and successfully established (Kalpana *et al.*, 2009).

2.6 Acclimatization

Rooted plantlets of *Stevia* transferred for hardening, with 90% of plantlets successfully established in the field was found by Rafiq *et al.* (2007).

Chotikadachanarong and Dheeranupattana (2013) found the high survival rate (80%) of *Stevia* was obtained when the rooted plantlets were transferred to greenhouse conditions.

Singh and Dwivedi (2013) conduct a research on *Stevia* to see that the survival of the hardened in vitro raised plants, under controlled condition was 90%. The field transplantation of these plants showed 70% survival after 2 months.

The maximum number of roots (30.12 ± 2.1 roots per explants) of *Stevia* was obtained on a MS medium containing 1.0 mg L^{-1} IBA. The well rooted plantlets were successfully weaned and acclimatized in plant soil with survival rate of 83.3 % (Razak *et al.*, 2014).

Shende and Manik (2013) treatment containing 1.50 mg/L BAP produced higher mean number of shoots per explants for rapid multiplication of *Stevia rebaudiana.* The regenerated shoots were successfully rooted on MS medium supplemented with 1.00 mg/L NAA. The rooted plantlets were acclimatized on sterilized Soil, Sand and Manure at 1:1:1 ratio. A total of 80% plants survived through this process.

The best in vitro root induction (89%) of *Stevia* was achieved on half strength MS medium without any growth regulator with an average of 24 roots per culture and root length of 7 cm. The rooted plantlets were successfully established in soil and grown to maturity at the survival rate of 95% in the indoor grow room (Lata *et al.*, 2013).

In vitro regenerated plants of *Stevia* were able to acclimate to greenhouse conditions in the first 15 days; after 60 days 81 % of plants survived, and four months later those plants produced seeds (Vazquez-Baxcajay, 2014).

Roots of *Stevia* produced within two weeks and the highest percentage (98.72%) of root induction. The *ex-vitro* plantlets were successfully acclimatized with a survival rate of 70% at the hardening phase (Soliman *et al.*, 2014).

Medium supplemented with 2.0 mg/L IBA showed highest rooting (69.76%) and early root initiation (7.1 days) of *Stevia*. Regenerated plantlets were successfully hardened and acclimatized in glass jar covered with poly propylene cap under net house conditions with 94.8 percent survival (Verma *et al.*, 2011).

Chapter III
Materials and Methods

CHAPTER III

MATERIALS AND METHODS

3.1 Time and location of the experiment

An experiment on *in vitro* propagation of *Stevia rebaudiana* were conducted in the Biotechnology laboratory of the Department of Biotechnology, Sher-e-Bangla Agricultural University (SAU), Sher-e-Bangla Nagar, Dhaka-1207, during the period of January, 2014 to December, 2014. Three experiments were conducted to fulfill the objectives of the present study.

Experiment 1 : Multiple shoot proliferation in *S. rebaudiana.*

1.1 : Effect of BA and KIN on multiple shoot proliferation.

1.2 : Effect of BA+IBA and KIN+IBA on multiple shoot proliferation through callus initiation.

Experiment 2 : Root formation in *S. rebaudiana.*

Experiment 3 : Acclimatization and establishment of plants in natural field condition.

3.2 Experimental materials

3.2.1 Source of material

The planting materials of *Stevia* were collected from local nurseries of Agargaon, Sher-e-Bangla Nagar, Dhaka -1207.

3.2.2 Plant materials

Disease free shoot tip and nodal segments of *Stevia rebaudiana* were used as explants in this experiment. The explants were washed thoroughly with running tap water for removing soil from root. Shoot with young leaves were collected from the Stevia plants (Plate 1A). The extra leaves were removed and shoot were trimmed to size of 1-2 cm for further use as explant (Plate 1B).

1. (A)

1. (B)

Plate 1. Explants collection from Stevia plant, (A) Stevia plant (B) Shoot tip and nodal segments

3.2.3 Instruments and glass ware

Instruments like; Forceps, scalpels, needless, spatulas, aluminum foils, tissue, cotton, plastic caps etc. were used as instruments and Erlenmeyer flasks, culture bottles, flat bottom flasks, pipettes, petridishes, beaker and measuring cylinders (25 ml, 50 ml, 100 ml, 500 ml and 1000 ml) etc. were used as glassware.

3.2.4 Culture media

The degree of success in tissue culture is mainly related to the choice of nutritional components and growth regulators. Presence of plant growth regulators plays a significant role in a successful regeneration of any plant species. Media for tissue culture should contain all major and minor elements, vitamins and growth regulators which are essential for normal plant growth. Explants were inoculated onto media composed of basal MS medium supplemented with the plant growth regulators. Composition of MS media have been shown in appendix I. Hormones were added separately to different media according to the requirements.

3.3 Sterilization of Instruments and Glass ware

All the glassware and instruments were first rinsed with the liquid detergent (Trix) and washed thoroughly with tap water until the detergent was removed completely.

Then the glassware and instruments were sterilized in an autoclave at a temperature of 121^0C and at 1.06 kg/cm^2 (15 PSI) pressure for 30 minutes. Oven dried (250^0C) were used for media preparation.

3.4 Preparation of stock solutions

The first step in the preparation of the medium was the preparation of stock solutions. As different ingredients were required in different concentrations, separate stock solutions for macronutrients, micronutrients, vitamins, growth hormones etc, were used.

3.4.1 Stock solution A (Macronutrients)

Stock solution of macronutrients was prepared up to 10 times the concentration of the final medium in 1000 ml of distilled water (dw). Ten times the weight of the salts required per litre of the medium were weighed properly and dissolved by using a magnetic stirrer in about 750 ml of distilled water and then made up to 1000 ml by further addition of distilled water (dw). To make the solution free from all sorts of solid contaminants, it was filtered through Whatman no. I filter paper. Then it was poured into a plastic container, labeled with marker and stored in a refrigerator at 4^0C for later use.

3.4.2 Stock solution B (Micronutrients)

The stock solution of micronutrients was made up to 100 times the final strength of necessary constituents of the medium in 1000 ml of distilled water (dw) as described for the stock solution of macronutrients. The stock solution was filtered, labeled and stored in a refrigerator at 4^0C.

3.4.3 Stock solution C (Iron sources)

This was prepared at 100 times the final strength of Fe_2SO_4 and Na_2EDTA in 100 ml of distilled water and chelated by heating on a heater cum magnetic stirrer. Then the volume was made up to 1000 ml by further addition of distilled water. Finally the stock solution was filtered and stored in a refrigerator at 4^0C.

3.4.4 Stock solution D (Vitamins)

Each of the desired ingredients except myo-inositol were taken at 10 folds (100x) of their final strength in a measuring cylinder and dissolved in 750 ml of distilled water. Then the final volume was made up to 1000 ml by further addition of distilled water. The solution was

dispensed into 10 ml aliquots and stored at -20^0C. Myo-inositol was used directly at the time of media preparation.

3.4.5 Hormone stock solution

The first step in the preparation of the medium was the preparation of hormone stock solutions. To expedite the preparation of the medium separate stock solutions for growth regulators were prepared and used. Growth regulators and concentrations used in this present investigation are presented in Table 1.

Table 1. Phytohormone used for *in vitro* regeneration of *Stevia*

Sl. No.	Growth regulators	Application for	Concentration (mg/L)
1.	BA	Shoot regeneration	0.5, 1.0, 1.5, 2.0, 2.5
2.	KIN	Shoot regeneration	0.5, 1.0, 1.5, 2.0, 2.5
3.	BA	Callus induction	2.0
4.	KIN	Callus induction	1.0
5.	IBA	Callus induction	0.5, 1.0, 1.5, 2.0
6.	IBA	Root induction	0.5, 1.0, 1.5, 2.0

The MS medium without any hormone used as a control for both the test. Sucrose (3%) was used as carbon source and media were solidified with agar-agar (0.8%).The pH was adjusted to pH 5.8 prior to autoclaving at a temperature of 121^0C for 20 minutes at 1.06 kg/cm^2 (15 PSI) pressure.

3.5 Preparation of the stock solution of hormones

To prepare the above hormonal supplements, they were dissolved in proper solvent as shown against each of them below. Generally, cytokinins were dissolved in few drops of basic solutions (1N NaOH) and auxins were dissolved in few drops of basic solutions (1N NaOH) or 70% ethyl alcohol.

Hormones (Solute)	**Solvents used**
BA	1 N NaOH
KIN	1 N NaOH
IBA	70% ethyl alcohol

In present experiment, the stock solution of hormones were prepared by following procedure.

3.5.1 Stock solution of BA and KIN: 100 mg of powder hormone was placed in a small beaker and then dissolved in 10 ml of 1 (N) NaOH solvent. Finally the volume was made up to 100 ml by the addition of sterile distilled water using a measuring cylinder.

3.5.2 Stock solution of IBA: 100 mg of powder hormone was placed in a small beaker and then dissolved in 10 ml of 70% ethyl alcohol solvent. Finally the volume was made up to 100 ml by the addition of sterile distilled water using a measuring cylinder The prepared hormone solution was then labeled and stored at 4 ± 1^{0}C for use up to two month.

3.6 Preparation of culture media from ready made MS powder

To prepare 1000 ml of culture media the following steps were followed:

Step-1. 700 ml of sterile distilled water was poured into 1000 ml beaker.

Step-2. 5 gm of MS media (readymade) and 30 gm of sucrose was added and gently stirred to dissolve these ingredients completely with the help of a hot plate magnetic stirrer.

Step-3. Different concentrations of hormonal supplements were added to the solution either in single or in combinations as required and mixed well.

Step-4. The volume was made up to 1000 ml with addition of sterile distilled water.

Step-5. The pH was adjusted at 5.8.

Step-6. Finally, 8 gm agar was added to the mixture and heated for 10 minutes in an electric oven for melting of agar.

3.7 Steam heat sterilization of media (Autoclaving)

For sterilization the culture medium was poured in 200 ml culture bottles and then autoclaving was done at a temperature of 121^{0}C for 20 minutes at 1.06 kg/cm^2 (15 PSI) pressure. After autoclaving the media were stored in at 25±2 °C for several hours to make it ready for inoculation with explants.

3.8 Sterilization of culture room and transfer area

In the beginning, the culture room was spray with formaldehyde and then the room was kept closed for one day. Then the room was cleaned through gently washing the floors, walls and rakes with detergent. This is followed by careful wiping them with 70% ethanol. This process of sterilization of culture room was repeated at regular intervals.

The transfer area was also cleaned with detergent and also sterilized twice in a month by 70% ethanol. Laminar airflow cabinet was usually sterilized by switching UV ray to kills the

microbes inside the laminar airflow. It switches on 30 minutes before working in empty condition and for 20 minutes with all the instruments. The working surface was wiping with 70% ethanol before starting the transfer work.

3.9 Preparation of explants

The explants (Shoot tip and nodal segments) were washed thoroughly under running tap water and then with autoclaved distilled water for several times.

Subsequently the explants were transferred to laminar airflow cabinet and kept in a 250 ml sterilized beaker. The beaker with explants was constantly shaken during sterilization. They were treated with 70% ethanol for 1-2 minute and rinsed with autoclave distilled water for 3-4 times. After treating with 70% ethanol, the explants were surface sterilized with a 0.1% mercuric chloride solution (plate 2) containing two-three drops of tween-20 for 5 min under aseptic condition and then washed four-five times with autoclaved distilled water to make the material free from chemical and ready for inoculation in culture media.

Plate 2. Sterilization of explants of *Stevia* plant material with 0.1% $HgCl_2$

3.10 Placement of explants into the media/Inoculation

For inoculation, the worker hands and forearms were washed thoroughly with soap or antiseptic and repeatedly sprayed with 70% alcohol during the period of work. Prior to use, the surface of the laminar flow bench was swabbed down with 70 % ethyl alcohol and the interior sprayed with same alcohol. All glassware, instruments and media were steam-sterilized in the autoclave. During the course of work, small instruments in use were placed in a beaker containing 70 % ethanol and were flamed repeatedly using a spirit burner. Explants

were transferred to large sterile glass petridish or glass plate with the help of sterile forceps under strict aseptic conditions. Here the explants were further trimmed and extra outer leaves were removed with sterile scalpel blade to make suitable size. The surface sterilized explants were inoculated carefully following proper sterilization process within laminar airflow cabinet. The mouth of culture vial was flamed before and after positioning of the explants on the medium.

After cutting explants into suitable size (1-2 cm), explants are transferred to culture bottles containing 20 mL MS medium with plant growth regulator (Plate 3). After vertically inoculating the explants singly in culture bottle, the mouth of bottle was quickly flamed and capped tightly. After proper labeling, mentioning media code, date of inoculation etc. the bottles was transferred to growth room.

Plate 3. Inoculation of culture with the explants of *Stevia rebaudiana* Bertoni

Some explants became black in color within 6-7 days after inoculation. To control blackening the blackish tissues on the explants were removed and the explants were transferred to similar fresh medium. It was repeated each of 10 days interval for about one month to minimize further blackening of the tissues.

3.11 Incubation

The culture vials then transferred to culture racks and allowed to grow in controlled environment. The temperature of the growth room was maintained within 25±1° C by an air conditioner and a 16 hour photo period was maintained along with light intensity of 2000 lux for proper growth and development of culture (Plate 4).

Plate 4. Incubation of inoculated culture vial

3.12 Subculturing and maintaining of proliferating shoots

Initial subculturing was done after 15 to 30 days when the explants had produced some shoots. For sub-culturing, the entire samples of *in vitro* shoot were cut into small pieces. Shoots were excised in aseptic condition with help of sterile scalpel blade and sterile forceps and transferred to new MS media which was supplemented with same concentration of growth hormones in order to increase budding frequency (Plate 5). The observations on development pattern of shoots were made throughout the entire culture period. Data recording was started after 2nd weeks from inoculation, so that each piece would contain about one shoot. Leaf and blackish or brownish basal tissues were removed. Each piece was inoculated into a similar fresh medium. It was practiced at the interval of 20-25 days.

Plate 5. Subculturing and maintaining of subcultured vial

3.13 Root formation of regenerated shoots

Newly formed shoots with adequate length were excised individually from the culture vial and transferred to rooting media. Growth regulators (IBA) was used in different concentration. Some roots produce from callus. The observations on development pattern of roots were made throughout the entire culture period. Data were recorded from 2nd week of inoculation.

3.14 Acclimatization

Acclimatization or "hardening-off' is a process by which *in vitro* propagated plants are made to adapt to an *in vivo* environment.

Step-1: After 25 to 30 days of culture on rooting media, the plantlets were taken out from culture vial with the help of forceps with utmost care to prevent any damage to newly formed roots and dipped in gentle warm water to remove any traces of solidified agar media for acclimatization. Plastic pots (6×6 cm) were kept ready filled with garden soil and compost in the proportion of 1:1 respectively. Immediately after removing solidified agar media from newly formed roots, the plantlets were then transplanted in to the pots with special care.

Step-2: After planting, the plantlets were thoroughly watered and were kept at 25±2°C with light intensity varied from 2000–3000 lux. The photoperiod was generally 14 hours light and 10 hours dark and 70% RH for 7 days with consecutive irrigation.

Step-3: Then the plants were shifted to shade house with less humidity and indirect sunlight. The top of the pots were covered with transparent plastic sheet and grew at room temperature and 70% RH for 14 days with periodic irrigation (2days intervals).

Step-4: After 3 weeks, the plants were transferred to the soil following depotting and potting into different pot having bigger pot size. The plants were watered periodically and upper layer of the soil mulched occasionally whenever necessary.

3.15 Data recording:

The observations on development pattern of shoots and roots were made throughout the entire culture period. Five replicates (single shoot per culture bottle) were used per treatment. Data were recorded after 2 and 4 weeks of culture, starting from day of inoculation on culture media in case of shoot proliferation. In event of root formation, it was done every week

starting from third week to fifth week of culture. The following observations were recorded in cases of shoot and root formation under *in vitro* condition.

1. Days for shoot induction
2. Days to callus induction
3. Days to initiate shoot from callus
4. No. of shoots per explants
5. Average Length of shoot (cm)
6. Length of longest shoot
7. No. of leaf per explants
8. Days to root induction
9. No. of roots per explants
10. Average length of root (cm)
11. Length of the longest root (cm)
12. Percent of explants showing shoot induction
13. Percent of explants showing root induction
14. Percent of explants showing callus induction

3.15.1 Calculation of days to shoot and root induction

Days to shoot and root induction were calculated by counting the days from explants inoculation to the first induction of shoot/root.

3.15.2 Calculation of number of shoots and roots per explants

Number of shoot and root per explants was calculated by using the following formula,

$$\text{Number of shoot / root per explants} = \frac{\text{Number of shoot / root per explants}}{\text{Number of observation}}$$

3.15.3 Calculation of number of leaves

Numbers of leaves produced on the plantlet were counted and the mean was calculated.

3.15.4 Calculation of shoot and root length (cm)

Shoot and root length were measured in centimeter (cm) from the base to the top of the explants by a measuring scale. Then the mean was calculated.

3.15.5 Calculation of percent of shoot and root induction from culture

Number of shoot and root were recorded and the percentage of shoot and root induction was calculated as:

$$\text{Percent (\%) of shoot induction} = \frac{\text{Number of explants induced shoot}}{\text{Number of explants incubated}} \times 100$$

The percentage of root induction was calculated as:

$$\text{Percent (\%) of root induction} = \frac{\text{Number of shoot induced root}}{\text{Number of shoot incubated}} \times 100$$

3.15.6 Calculation of percent of explants induce callus from culture

Percentage of explants showing callus induction was calculated as:

$$\text{Percent (\%) of explants showing callus induction} = \frac{\text{Number of explants induced callus x100}}{\text{Number of explants incubated}}$$

3.15.7 Calculation of percent of established plantlet:

The percentage of established plants was calculated based on the number of plantlets placed in the pot and the number of plants finally established or survived by the following equation:

$$\text{Percentage (\%) of established plantlets} = \frac{\text{Number of established plantlet}}{\text{Total number of plantlets}} \times 100$$

3.16 Statistical analysis

The experiment was one factorial set up in a completely randomized design (CRD) with five replications per treatment. Data were statistically analyzed by analysis of variance (ANOVA) technique and differences among treatment means were compared by using Least Significant Difference test (LSD) at 5% probability level using Statistix-10 (2013) program.

Chapter IV
Results and Discussion

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Experiment 1. Multiple shoot proliferation in *S. rebaudiana*

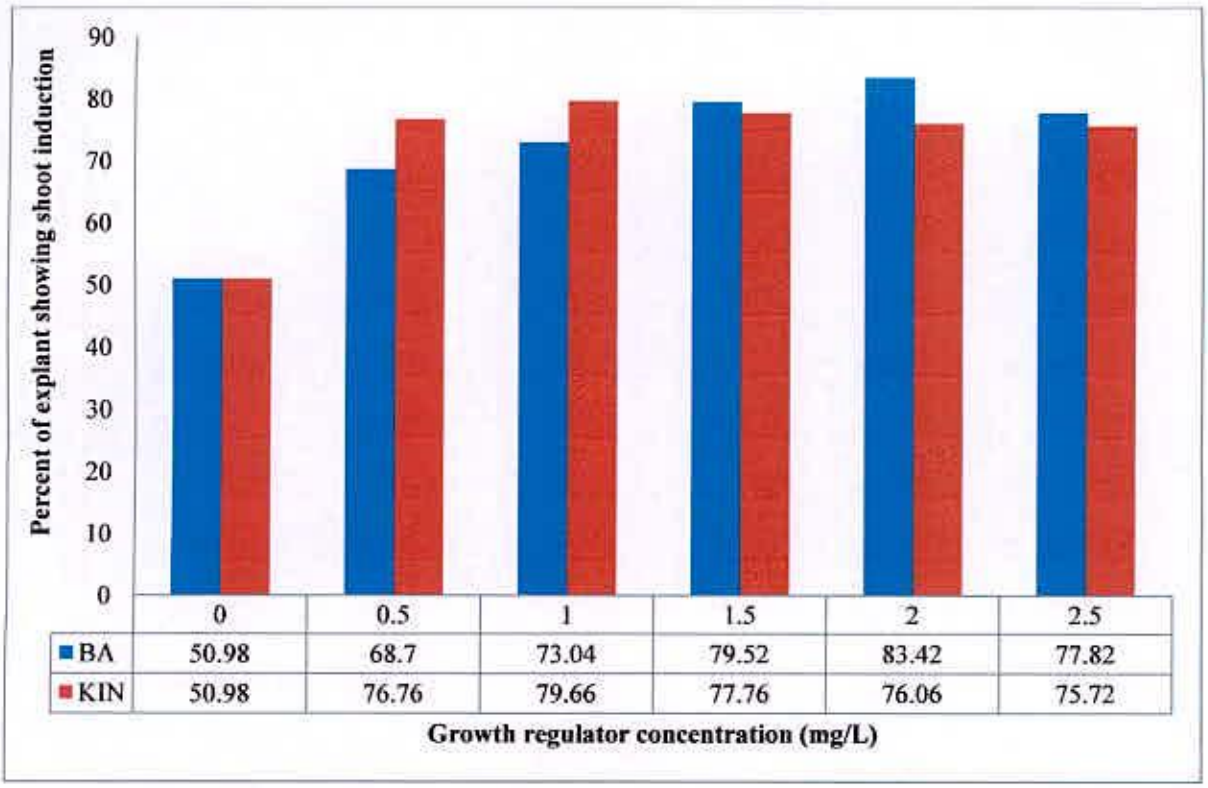
This experiment was conducted under laboratory condition to evaluate the effect of different plant growth regulators on shoot proliferation. Manipulating the relative ratio of different growth regulators has been successfully used in the current investigation. BA and KIN alone used for direct multiple shoot proliferation. On the other hand BA+IBA and KIN+IBA used for callus induction and shoot regeneration. The results are presented separately under different headings below.

4.1.1 Effect of BA and KIN on multiple shoot proliferation.

The results of the effect of different concentrations of BA and KIN have been presented under following headings with Figures 1-6; Table 2 and Plate 6-9.

4.1.1.1 Percent of explants showing shoot induction

There was a significant variation on percentage of explants showing shoot induction in presence of various concentrations of BA and KIN at 5% level of significance. The highest percentage (83.42%) of shoot induction was recorded at 2.0 mg/L and in case of 1.0 mg/L KIN highest percentage (79.66%) was recorded. The lowest percentage (50.98%) was induced in hormone free media in *Stevia* (Figure 1). Maximum shoot multiplication response (90%) was obtained in MS medium having 1.0 mg/L of BAP, with average of 8.6 shoots per culture vial having an average shoot length of 6.0 cm (Ali *et al.*, 2010).When the explants were culture on 0.25 to 1.50 mg/L BAP containing media 70-80% of them produced shoots is reported by Shende and Manik (2013). Maximum multiple shoots (96%) were obtained in MS medium supplemented with 1.0 µM TDZ (thidiazuron) with an average of 60 shoots per culture, having an average shoot length of 6.0 cm (Lata *et al.*, 2013).



	0	0.5	1	1.5	2	2.5
BA	50.98	68.7	73.04	79.52	83.42	77.82
KIN	50.98	76.76	79.66	77.76	76.06	75.72

Figure 1. Effects of BA and KIN on percent of explants showing shoot induction.

4.1.1.2 Days to shoot induction:

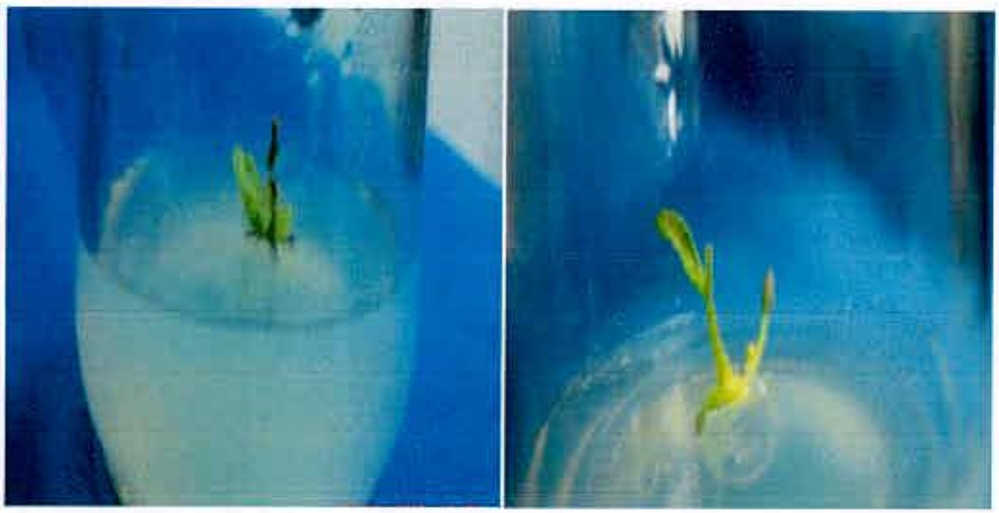
Significant variations were observed among different concentration of Kinetin on days to shoot induction. The maximum days to shoot induction were recorded in control (18.4 days) and in case of 1.0 mg/L KIN required minimum 11 days and BA 2.0 mg/L required minimum 10.6 days (Table 2 & plate 6).

BAP has been considered to be most effective for the induction of shoot in plant tissue cultur e(Asamenew and Narayanaswamy, 2000; Baskaran and Jayabalan, 2005; Russell, 1979).

Table 2. Effect of BA and KIN on days to shoot induction in *S. rebaudiana.*

Combination of growth regulator	Concentration mg/L	Days to shoot induction
Control	0	18.4 a
BA	0.5	13.8bc
BA	1	13.8bc
BA	1.5	11.4e
BA	2	10.6e
BA	2.5	13.4cd
KIN	0.5	13.0cd
KIN	1	11.0e
KIN	1.5	12.0de
KIN	2.0	14.2bc
KIN	2.5	15 b
CV (%)		8.70
SE		0.7336
$LSD_{(0.05)}$		1.4785

In a column, means having similar letter(s) are statistically similar and those having dissimilar letter(s) differ significantly as per 0.05 level of probability.



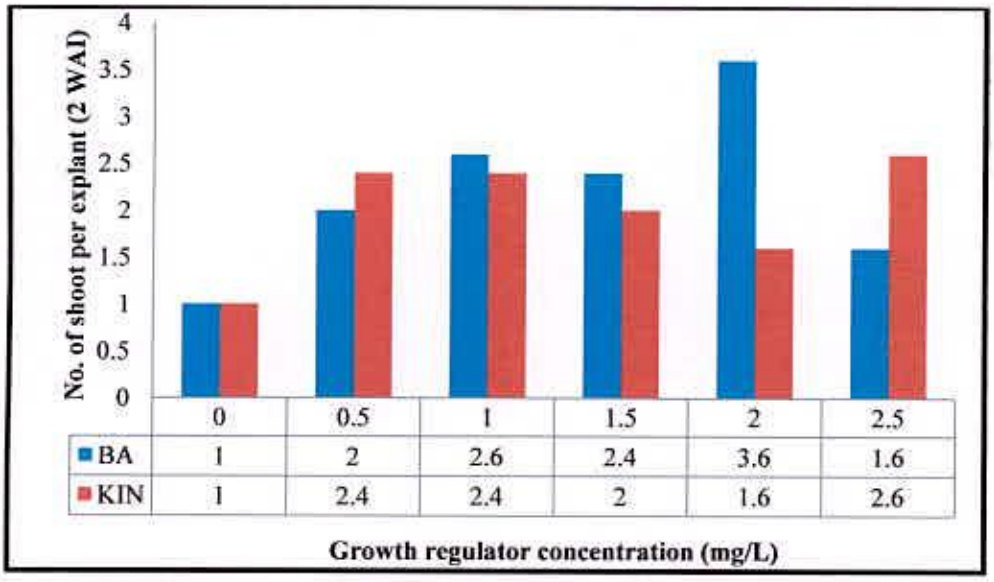
6. (A) **6. (B)**

Plate 6. Initiation of shoot at 2 WAI in the treatment of (A) 2.0 mg/L BA (B) 1.0 mg/L KIN

4.1.1.3 Number of shoots per explants at 2 WAI:

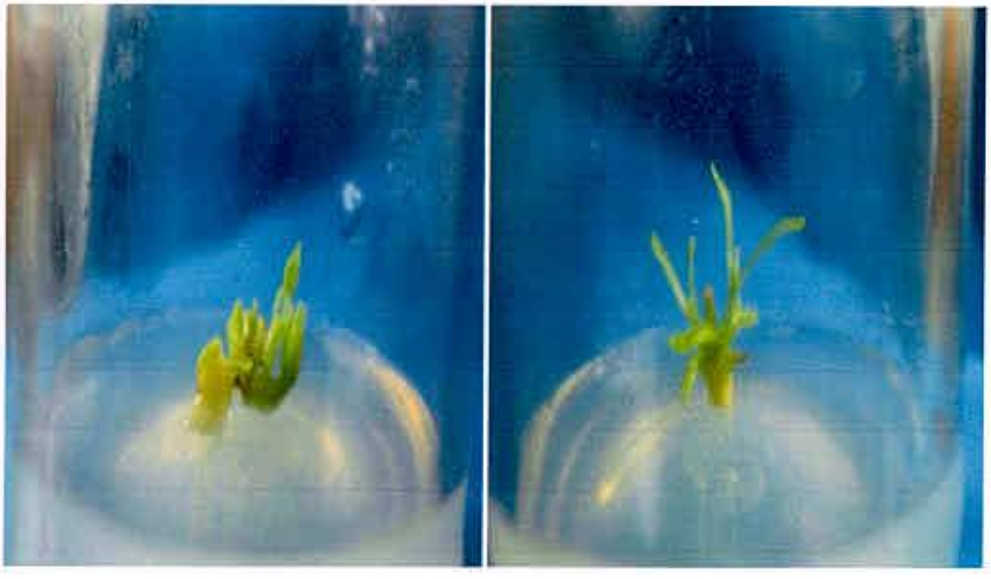
There was significant influence of different concentrations of Kin and BA on the number of shoots per explants. Data were recorded after 2 weeks of culture on MS media. The results have been presented in Figure 2 and Plate 7. Here BA showed better performance than KIN. 2 mg/L BA produce maximum 3.6 shoots per explants at 2 WAI, where 2.5 mg/L KIN

produce maximum 2.6 shoots at 2 WAI. In both cases lowest number of shoots was found with hormone free media. Rafiq *et al.* (2007), no specific increase in multiple shoot formation occurred when the explants were cultured on KIN based media.



	0	0.5	1	1.5	2	2.5
BA	1	2	2.6	2.4	3.6	1.6
KIN	1	2.4	2.4	2	1.6	2.6

Figure 2. Effects of BA and KIN on no. of shoots per explants 2 WAI



7. (A) 7. (B)

Plate 7. Shoot proliferation of *S. rebaudiana* on MS media at 2 WAI. supplemented with (A) 2.0 mg/L BA and (B) 2.5 mg/L KIN.

4.1.1.4 Number of shoots per explants at 4 WAI

The various concentrations of the hormones used in this study induced the proliferation of multiple shoots derived from the nodal segments of *S. rebaudiana*. This data were collected at 4 WAI. Here KIN was less effective at inducing multiple shoots compared to BA. 2.0

mg/L BA produce maximum 10.8 shoots at 4 WAI, in contrast; KIN, 1.0 mg/L produce maximum 10.2 shoots at 4 WAI.Minimum shoots (1.6 shoots/explants) was found at 4 WAI in control in case of both hormone (Figure 3; Plate 8).The highest shoots production was found in the MS medium supplemented with 0.5 mg L^{-1} BAP and 0.25 mg L^{-1}Kn (7.82 ± 0.7) after four weeks of cultivation (Razak *et al.*, 2014).According to Tadhani *et al.* (2006), the maximum number of shoots were achieved on MS medium supplemented with 0.6 mg L^{-1} of BAP.

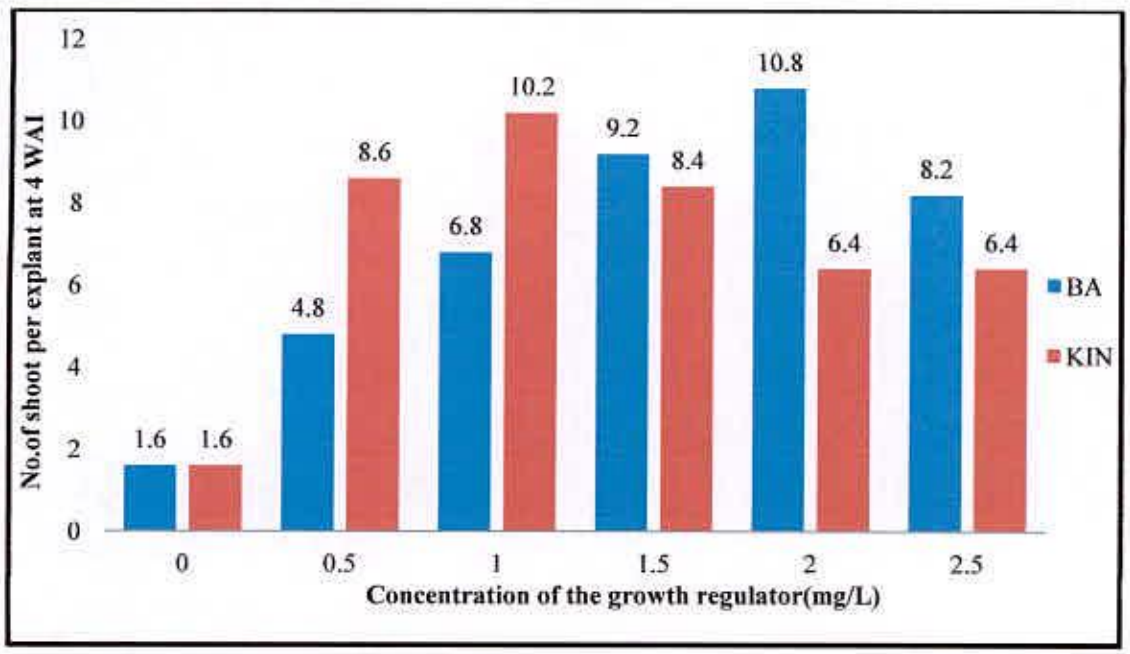


Figure 3.Effects of BA and KIN on no. of shoots per explants 4WAI

8. (A) **8. (B)**

Plate 8. Shoot proliferation of *S. rebaudiana* on MS media at 4 WAI at the treatment of (A) 2.0 mg/L BA and (B) 2.5 mg/L KIN

4.1.1.5 Number of leaves per explants

With different concentrations of Kinetin, significant variation was found on the number of leaves. The highest 17.2 leaves were recorded with 2.0 mg/L BA and highest 15.4 leaves were recorded with 1.0 mg/L KIN at 4 WAI. The lowest was found 6.6 in case of lack of hormone (Figure 4).

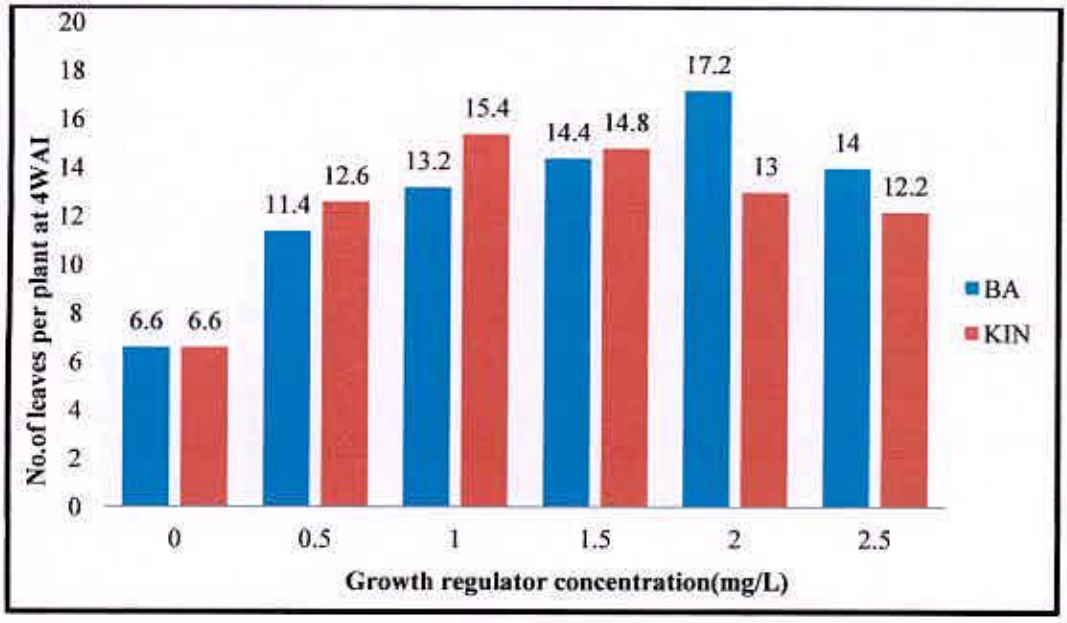


Figure 4. Effects of BA and KIN on number of leaves per explants 4WAI

4.1.1.6 Average length of shoot (cm) at 4WAI

The results of average length of shoot have been presented in Figure 5. The highest average length of shoot (3.94 cm) was noticed from the 1.0 mg/L KIN and highest (3.72 cm) at 2.0 mg/L BA at 4WAI. The minimum 2.1 cm was found in control (Figure 6).

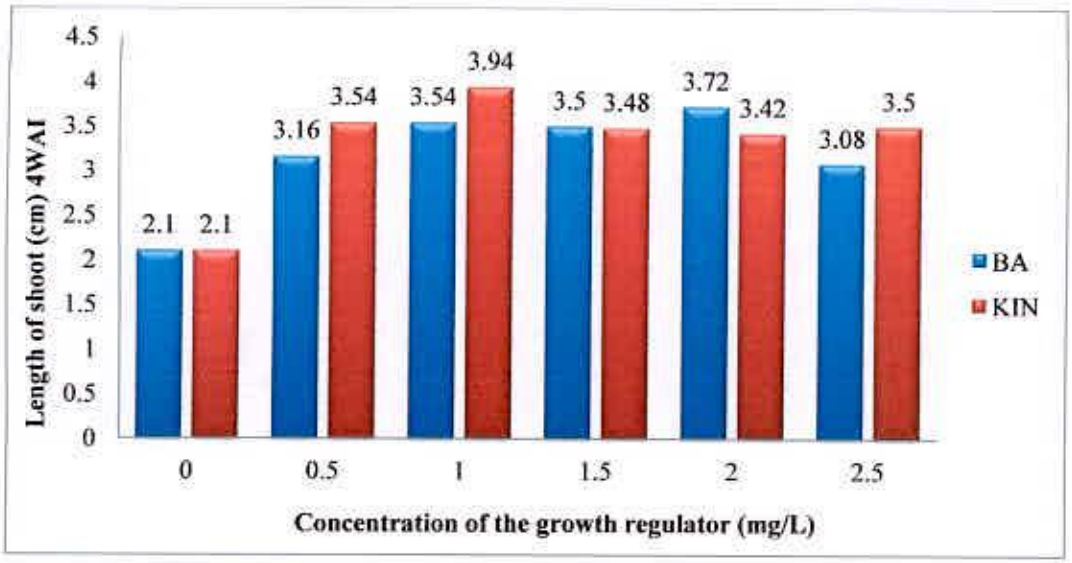


Figure 5. Effects of BA and KIN on average length of shoot (cm) at 4 WAI

4.1.1.7 Length of longest shoot (cm) 4WAI

Among the two cytokinin hormone longest shoot (4.20 cm) was found at 2.0 mg/L BA longest (4.16 cm) shoot was recorded at 1.0 mg/L KIN after 4 weeks of inoculation. Minimum longest shoot was recorded (2.34 cm) at control at 4 WAI (Figure 6 and Plate 9).

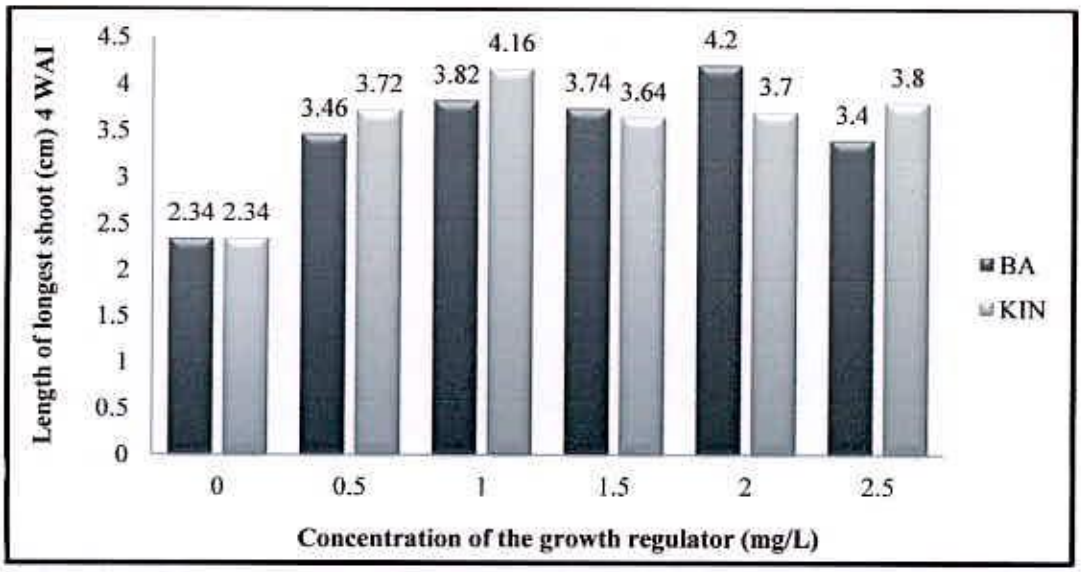
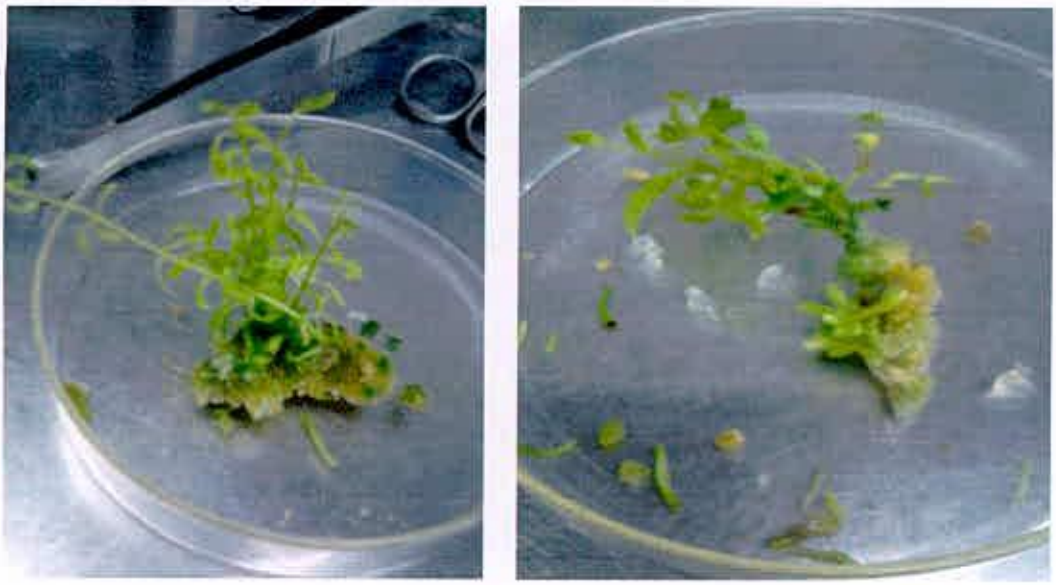


Figure 6.Effects of BA and KIN on length of longest shoot (cm) 4WAI



9. (A) **9. (B)**

Plate 9. Longest shoot produced supplemented with (A) 2.0 mg/L BA and (B) 1.0 mg/L KIN

4.1.2 Effect of BA+IBA and KIN+IBA on multiple shoot proliferation through callus initiation

The results of the effect of different concentrations of BAP have been presented under following headings with Table 3-7 and Plate 10-12.

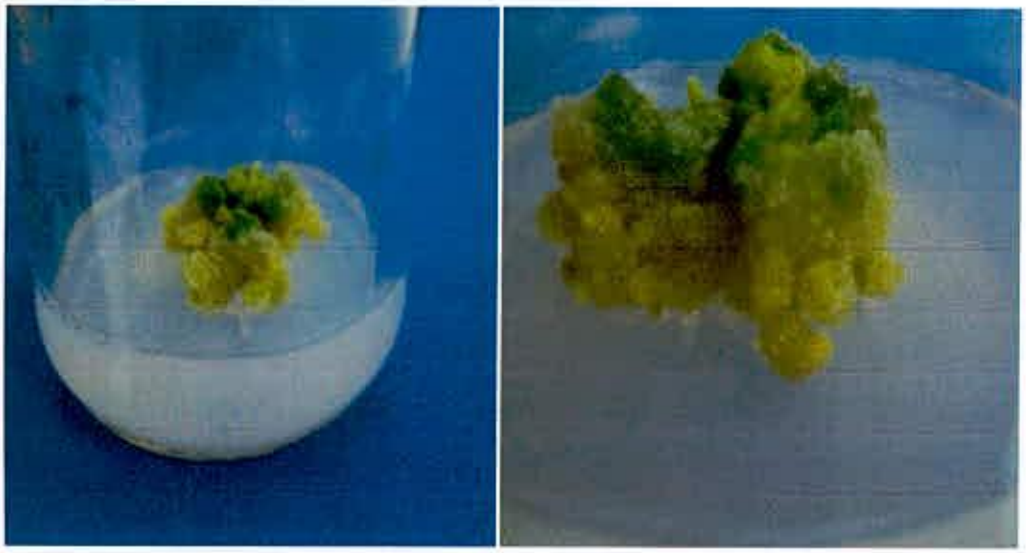
4.1.2.1 Days to callus induction:

There are two types of growth regulators with different combination used for callus induction. BA or KIN along with IBA used for callus induction. Minimum days (14) to shoot induction from callus was recorded at the treatment of 2.0 mg/L BA + 1.5 mg/L IBA. It was minimum (13.4) days for the treatment of 1.0 mg/L KIN + 1.5 mg/L IBA (Table 3 and Plate 10). MS media supplemented with NAA 2.0 mg/L +BA 2.0 mg/L was regarded as best hormonal treatment for callus induction from nodal segment (Ali *et al.*, 2010). Sivaram & Mukundan (2003) derived callus from on different combination of auxin and cytokinins like BA with IAA, IBA or 2,4-D and kinetin with NAA, IAA, IBA or 2,4-D. Swanson *et al.* (1992) observed the friable callus cultures from leaf explants of *Stevia rebaudiana* Bertoni cultured on MS medium supplemented with 0.5 mg/L of NAA, and 0.5 mg /L BAP.

Table 3. Combined effect of BA+IBA and KIN +IBA on days to callus induction in *S. rebaudiana.*

Combination of growth regulator	Concentration (mg/l)	Days to callus induction
BA+IBA	2.0+0.5	15.6 abc
BA+IBA	2.0+1.0	14.6 de
BA+IBA	2.0+1.5	14.0 ef
BA+IBA	2.0+2.0	15.2bcd
KIN+IBA	1.0+0.5	14.8cde
KIN+IBA	1.0+1.0	16.4a
KIN+IBA	1.0+1.5	13.4f
KIN+IBA	1.0+2.0	15.8ab
CV (%)		5.17
SE		0.4899
$LSD_{(0.05)}$		0.9979

In a column, means having similar letter(s) are statistically similar and those having dissimilar letter(s) differ significantly as per 0.05 level of probability.



10. (A) 10. (B)

Plate 10. Callus produced by (A) KIN+IBA in (1.0+1.5) mg/L and (B) BA+IBA in (2.0+1.5) mg/L

4.1.2.2 Days to shoot induction from callus

Minimum days (9.6) to shoot induction from callus was recorded at the treatment of 2.0 mg/L BA + 1.5 mg/L IBA. It was minimum (11.4) days for the treatment of 1.0 mg/L KIN + 1.5 mg/L IBA (Table 4 and Plate 11). Sivaram & Mukundan (2003) observed maximum shoot induction from shoot apex, nodal and leaf explants on Murashige & Skoog (MS) medium supplemented with 8.87 µM of BA and 5.71 µM of IAA. The nodal and inter-nodal explants showed maximum callus induction response i.e., 90 % (within 9 days of inoculation) and 73 % (within 7.3 days of inoculation) respectively on the MS medium supplemented with 3.0 mg/L NAA + 1.0 mg/L BAP (Ali *et al.*,2010).

Table 4. Combined effect of BA +IBA and KIN +IBA on days to shoot induction from callus of *S. rebaudiana.*

Combination of growth regulator	Concentration (mg/l)	Days to shoot induction from callus
BA+IBA	2.0+0.5	13.2 b
BA+IBA	2.0+1.0	11.0 c
BA+IBA	2.0+1.5	9.6 d
BA+IBA	2.0+2.0	13.8 ab
KIN+IBA	1.0+0.5	12.8 b
KIN+IBA	1.0+1.0	14.8 a
KIN+IBA	1.0+1.5	11.4 c
KIN+IBA	1.0+2.0	13.0b
CV (%)		6.6
SE		0.5194
$LSD_{(0.05)}$		1.0584

In a column, means having similar letter(s) are statistically similar and those having dissimilar letter(s) differ significantly as per 0.05 level of probability.

11. (A) 11. (B)

Plate 11. Shoot induction from callus at the treatment of (A) (1.0+1.5) mg/L KIN+IBA and (B) (2.0+1.5) mg/L BA+IBA

4.1.2.3 Number of shoot per callus 2 WAI

Maximum number of shoot was found after 2 weeks of inoculation (2.6) per callus was recorded at the treatment of 2.0 mg/L BA + 1.5 mg/L IBA. It was also maximum (2.6) for the treatment of 1.0 mg/L KIN + 1.5 mg/L IBA (Table 5).

Table 5. Combined effect of BA +IBA and KIN +IBA on number of shoot per callus 2 WAI of *S. rebaudiana.*

Combination of growth regulator	Concentration (mg/L)	Number of shoot per callus 2WAI
BA+IBA	2.0+0.5	2.4 a
BA+IBA	2.0+1.0	2.4 a
BA+IBA	2.0+1.5	2.6 a
BA+IBA	2.0+2.0	1.6 b
KIN+IBA	1.0+0.5	1.6 a
KIN+IBA	1.0+1.0	2.0 ab
KIN+IBA	1.0+1.5	2.6 a
KIN+IBA	1.0+2.0	1.4 b
CV (%)		25.91
SE		0.3606
$LSD_{(0.05)}$		0.7344

In a column, means having similar letter(s) are statistically similar and those having dissimilar letter(s) differ significantly as per 0.05 level of probability.

4.1.2.4 Number of shoot per callus 4WAI

After 4 weeks of inoculation maximum shoot number (10.4) per callus was recorded in the treatment of 2.00 mg/L BA + 1.5 mg/L IBA. It was recorded maximum (9.4) shoot per callus for the treatment of 1.00 mg/L KIN + 1.5 mg/L IBA (Table 6; Plate 12).

Table 6. Combined effect of BA +IBA and KIN +IBA on number of shoot per callus 4 WAI of *S. rebaudiana.*

Combination of growth regulator	Concentration (mg/L)	Number of shoot per callus 4 WAI
BA+IBA	2.0+0.5	7.2de
BA+IBA	2.0+1.0	7.6de
BA+IBA	2.0+1.5	10.4a
BA+IBA	2.0+2.0	6.8 e
KIN+IBA	1.0+0.5	8.0cd
KIN+IBA	1.0+1.0	8.2cd
KIN+IBA	1.0+1.5	9.4ab
KIN+IBA	1.0+2.0	8.8bc
CV (%)		9.71
SE		0.5099
$LSD_{(0.05)}$		1.0386

In a column, means having similar letter(s) are statistically similar and those having dissimilar letter(s) differ significantly as per 0.05 level of probability.

12. (A) 12. (B)

Plate 12. Number of shoot per callus 4WAI at the treatment of (A) (1.0+1.5) mg/L KIN+IBA and (B) (2.0+1.5) mg/L BA+IBA

4.1.2.5 Percent of explant showing callus induction

Maximum percentage (83.58%) of explants showing callus induction was recorded in 2.00 mg/L BA + 1.5 mg/L IBA. It was recorded maximum percentage (82.02%) for the treatment of 1.0 mg/L KIN + 1.5 mg/L IBA (Table 7).

Table 7.Combined effect of BA +IBA and KIN +IBA on percent of explants showing callus induction in *S. rebaudiana.*

Combination of growth regulator	Concentration (mg/L)	Percent of explant showing callus induction
BA+IBA	2.0+.5	79.08 cd
BA+IBA	2.0+1.0	80.48 c
BA+IBA	2.0+1.5	83.58 a
BA+IBA	2.0+2.0	80.08 cd
KIN+IBA	1.0+0.5	77.2 e
KIN+IBA	1.0+1.0	78.84 d
KIN+IBA	1.0+1.5	82.02 b
KIN+IBA	1.0+2.0	79.96
CV (%)		1.39
SE		0.7052
$LSD_{(0.05)}$		1.4364

In a column, means having similar letter(s) are statistically similar and those having dissimilar letter(s) differ significantly as per 0.05 level of probability.

4.2 Experiment 2. Root formation in *Stevia rebaudiana* B.

To develop root, the regenerated shoot excised and transferred to rooting media supplemented with IBA. Simultaneously along with callus and shoot, combination of BA+IBA and KIN+IBA also produce root. The results of experiment have been presented under different heading utilizing Figures 7-12, and Plates 13-16.

4.2.1 Percent of explant showing root induction

There were considerable variation among growth regulators on percent of explant showing root induction. The results have been shown in Figure 7. The highest percentage (78.18%) of root induction was recorded with 1.0 mg/L IBA when it applied singly. For combination highest percentage (76.92%) root induction was recorded by the treatment 1.0 mg/L KIN + 0.5 mg/L IBA. Lowest percentage (21.46%) of root induction was recorded in media lack of plant growth regulators. Slavova *et al.*, (2003) obtained 84% to 99% rooting on MS medium supplemented with NAA. Hwang (2006) reported the maximum numbers of roots using a treatment of 1.0 mg/L IBA in the MS medium with up to 100 % rooting. The maximum rooting response achieved on medium supplemented only with 2.46 µM IBA was 100% (Janarthanam *et al.*, 2009).

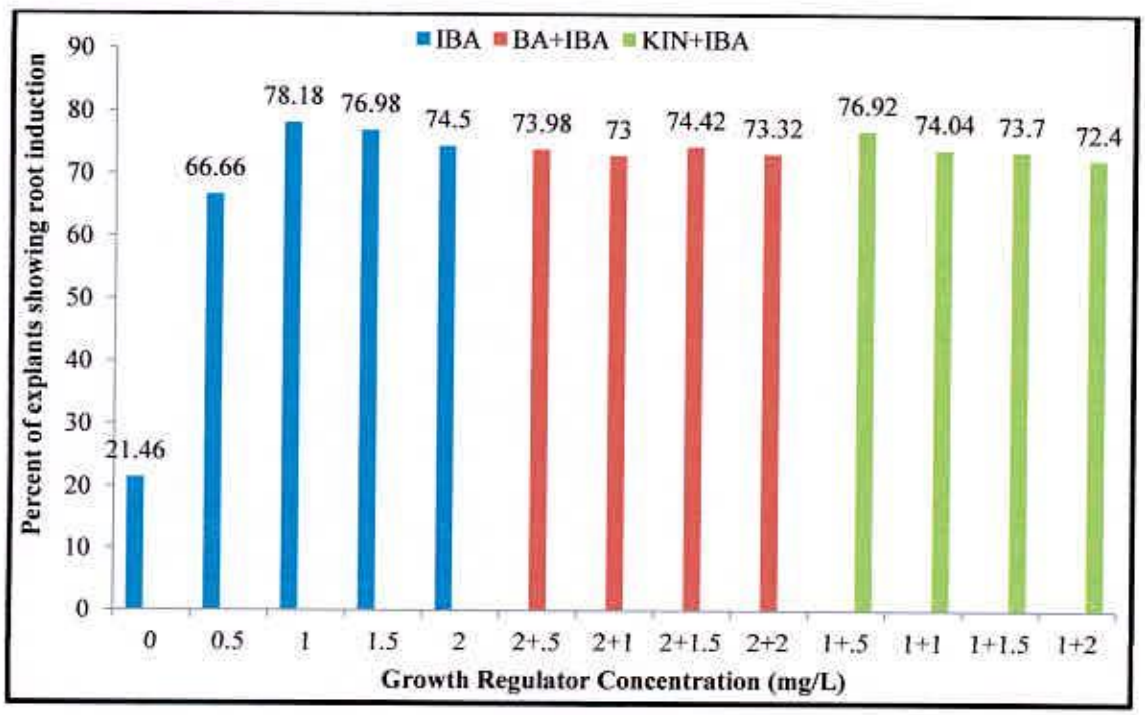


Figure 7: Effects of IBA, BA+IBA and KIN+IBA on percent of explant showing root induction

4.2.2 Days to root induction

Hormonal concentration has significant level of variation on days to root induction. Minimum 12.6 days in case of 1 mg/L IBA, on the other hand between BA+IBA and KIN+IBA minimum 13.4 days was recorded by the treatment 1.0 mg/L KIN + 0.5 mg/L IBA. The maximum 25.2 days to root induction was required in media lack of growth regulator (Figure 8; Plate 13). Developed shoots from nodal cuttings of *Stevia rebaudiana*, upon transfer to the MS medium containing indole butyric acid (IBA) at $0.1mgl^{-1}$ resulted in best rooting within 8 days (Karim *et al.*, 2008).

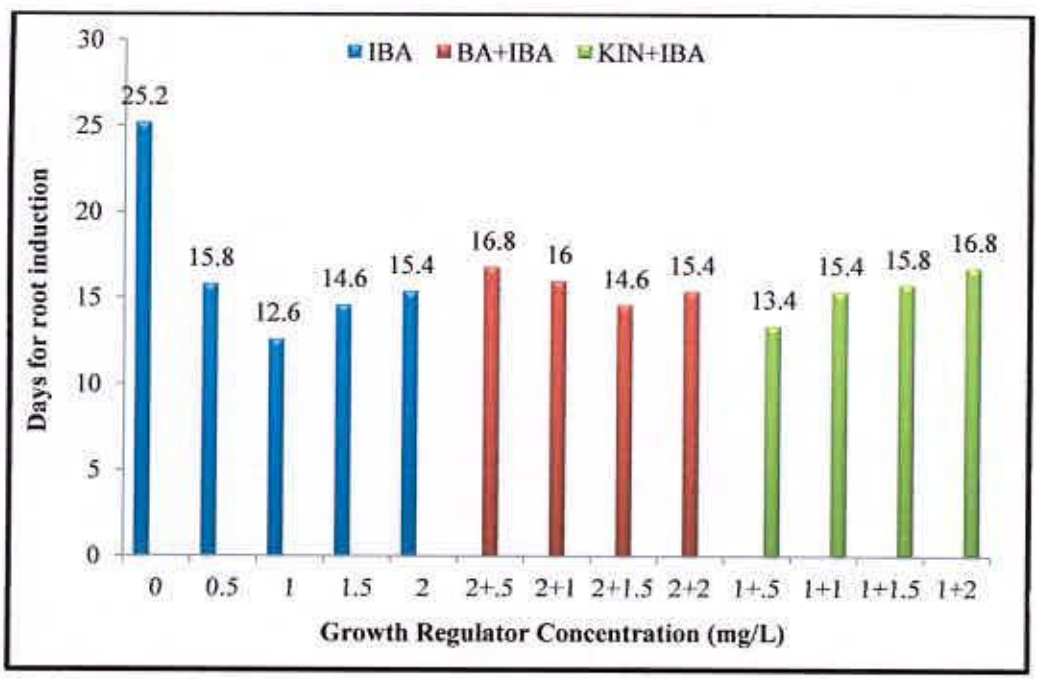
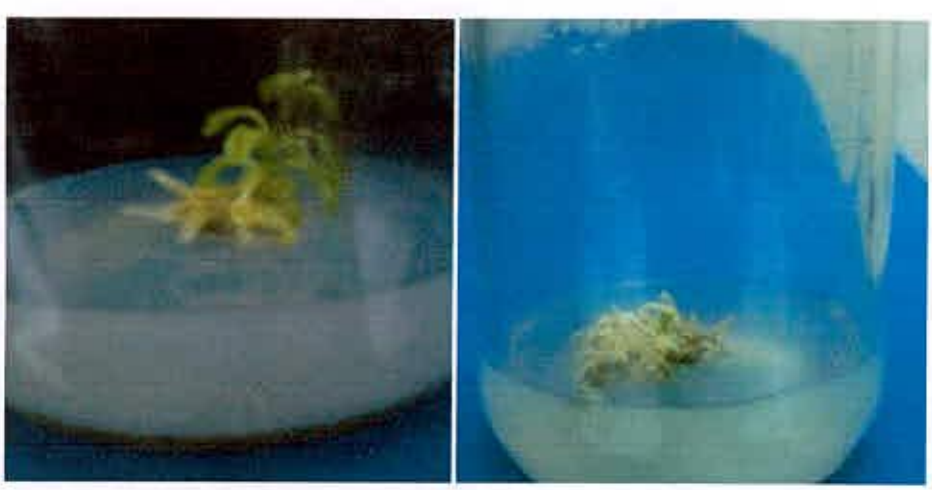


Figure 8: Effects of IBA, BA+IBA and KIN+IBA on days to root induction



13. (A) **13. (B)**

Plate 13. Induction of root by (A) 1.0 mg/L IBA, (B) (1.0+0.5) mg/L KIN+IBA

4.2.3 Number of roots per explants 2 WAI

The highest number of roots (4) per explants was recorded in 1.0 mg/L IBA at 2 WAI. In case of combination between BA+IBA and KIN+IBA, highest number of roots (4) was recorded by the treatment 1.0 mg/L KIN + 0.5 mg/L IBA. The minimum number of shoots (1.4) after 2 weeks was recorded in control i.e. at hormone free medium (Figure 9 and Plate 14). Tadhani *et al.* (2006) also obtained the highest rate of root induction in 1.0 mg/L IBA medium.The maximum rooting response achieved on medium supplemented only with 2.46 µ M IBA was an average of 7.3 ± 0.25 roots per shoot (Janarthanam *et al.*, 2009).

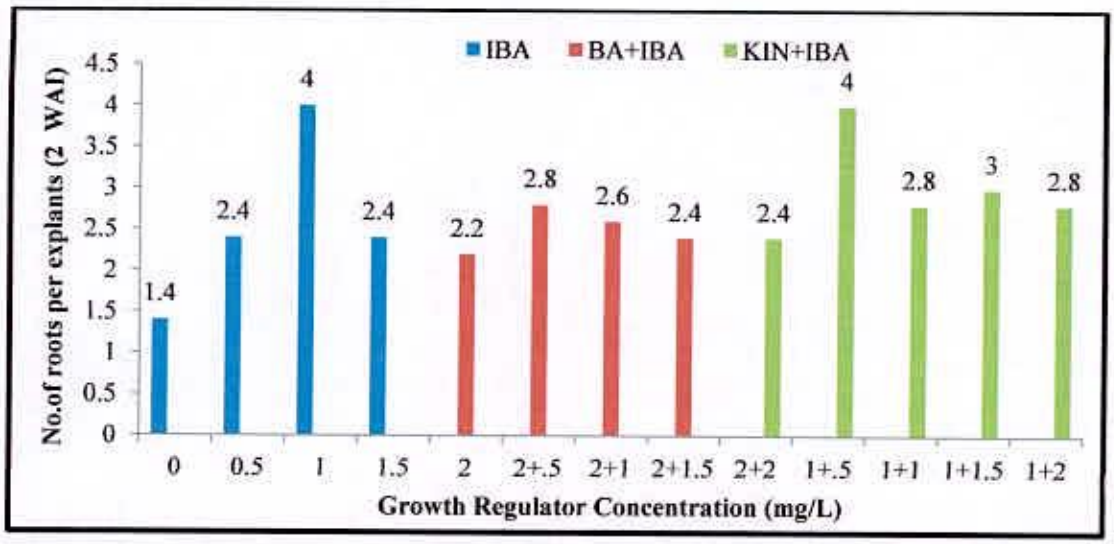


Figure 9: Effects of IBA,BA+IBA and KIN+IBA on number of roots per explants 2 WAI

14. (A) **14. (B)**

Plate 14.Number of roots per explants 2 WAI in MS medium supplemented with (A) 1.0 mg/L IBA and (B) (1+0.5) mg/L KIN+IBA

4.2.4 Number of roots per explants 4 WAI

In case of IBA alone, the highest number of roots (7.2) per explants was recorded in 1.0 mg/L IBA at 4 WAI. In case of combination highest number of roots (7.2) was recorded by the treatment (1.0 mg/L KIN + 0.5 mg/L IBA. Minimum number of roots (2.4) after 4 weeks was recorded in control (Figure 10; Plate 15).The maximum number of roots induced was observed in the MS medium supplemented with 1.0 mg L^{-1} of IBA (Razak *et al*, 2014). The maximum number of roots (9.46roots/shoot) was recorded on MS medium fortified with 0.5 mg/L IAA and 1.5 mg/L IAA (Soliman *et al.*, 2014). The best in vitro root induction with an average of 24 roots per culture was achieved on half strength MS medium without any growth regulator (Lata *et al.*, 2013).

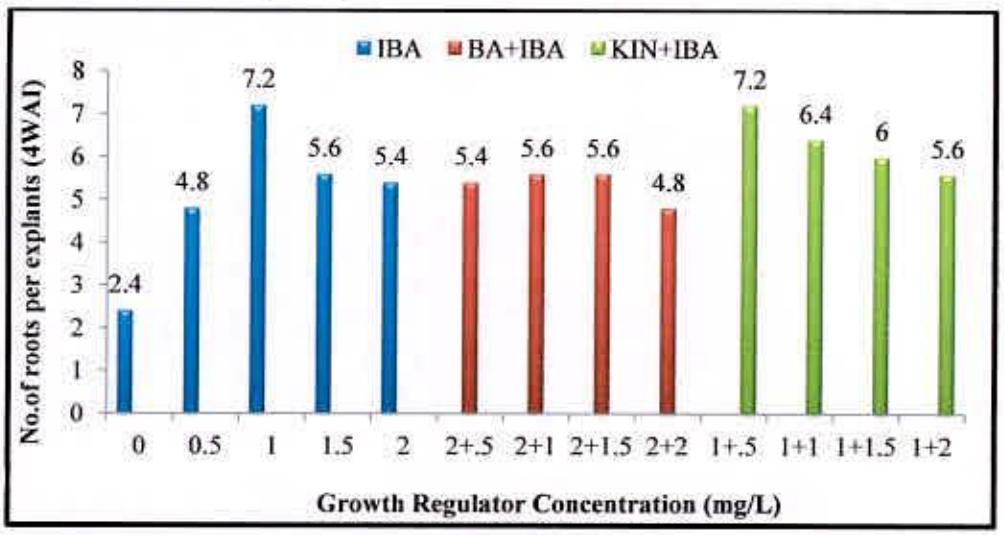


Figure 10: Effects of IBA,BA+IBA and KIN+IBA on number of roots per explants 4 WAI

15.(A) 15.(B)

Plate 15. Number of roots per explants 4 WAI in MS medium supplemented with (A) 1.0 mg/L IBA and (B) (1+0.5) mg/L KIN+IBA

4.2.5 Average length of root (cm)

Average length of roots per explants (cm) was greatly regulated by the different concentration of hormone. The maximum average root length (3.96 cm) was obtained from 1.0 mg/L IBA. In case of combination maximum root length (3.64 cm) was recorded by the treatment (1.0 mg/L KIN + 0.5 mg/L IBA. The minimum 1.32 cm average length of roots per explants (cm) was found in control (Figure 11). Root length was decreased in IBA containing medium and this was accentuated as the IBA concentration increased (Trauttman and Visser 1990).

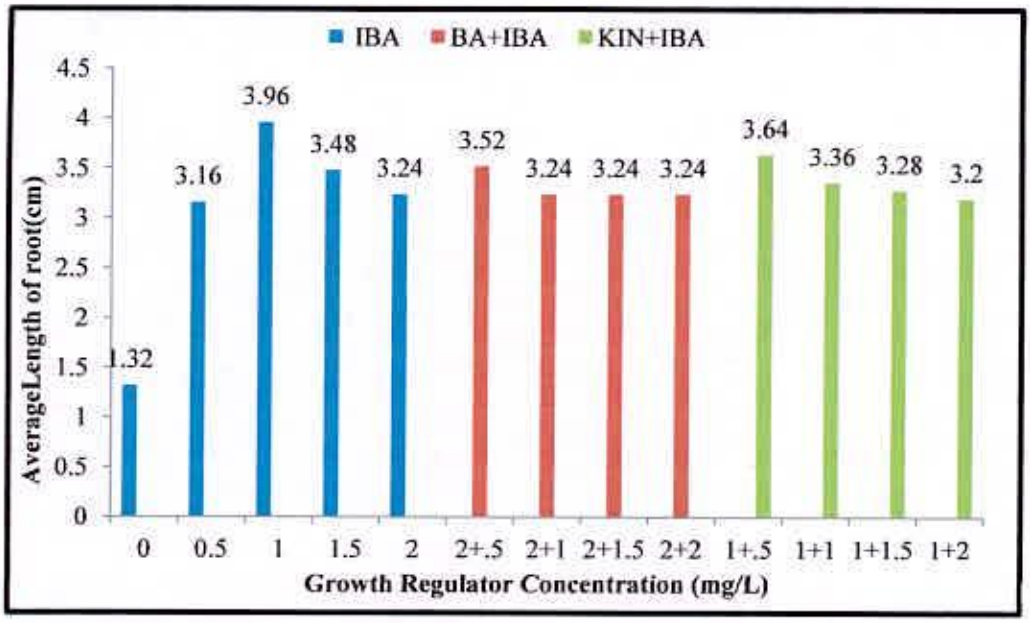


Figure 11: Effects of IBA, BA+IBA and KIN+IBA on average length of root(cm)

4.2.6 Length of longest roots (cm)

Different treatments of IBA applied alone, and IBA with shooting hormone play a vital role on length of longest roots (cm). The length of longest root (4.18 cm) was recorded with 1 mg/L IBA. In case of combination longest root (3.94 cm) was recorded by the treatment (1.0 mg/L KIN + 0.5 mg/L IBA. The least root length (1.56 cm) was reported from growth hormone free culture media (Figure 12 and Plate 16).The maximum root length (9.87 cm) was recorded on MS medium fortified with 1.5 mg/L IAA, respectively (Soliman *et al.*, 2014).

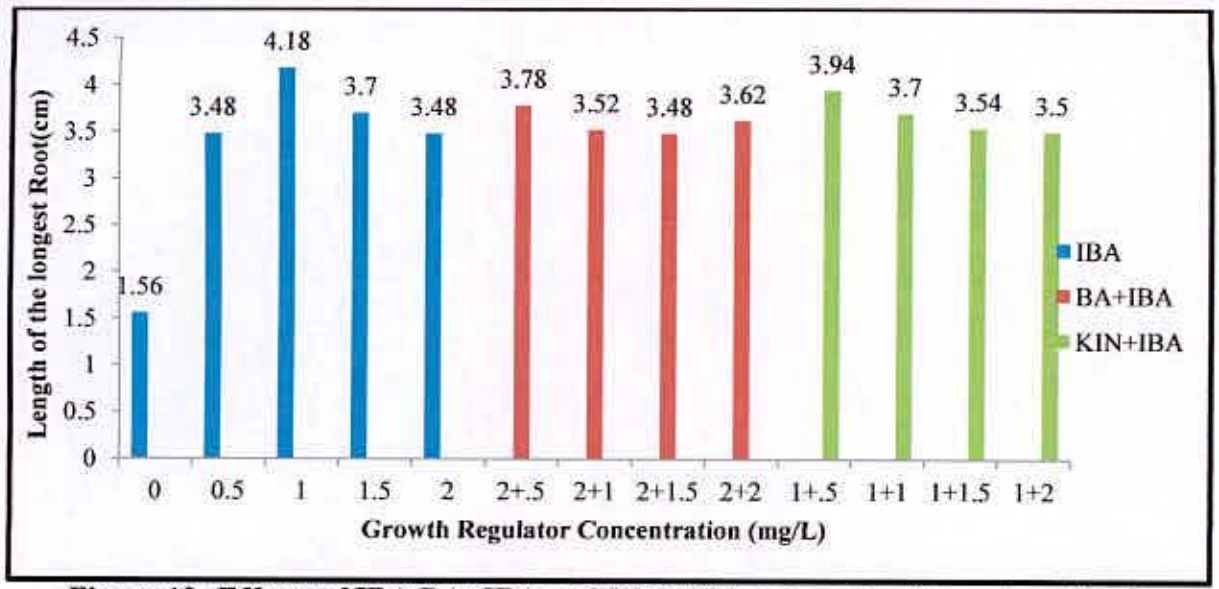


Figure 12: Effects of IBA,BA+IBA and KIN+IBA on length of longest root(cm)

Plate 16: The longest root of *Stevia* produced on MS media supplemented with 1.0 mg/L IBA

4.3 Experiment 3. Acclimatization and establishment of plants in natural field condition

After sufficient shoot and root development at 4 weeks of culture (Plate 17 A and 17 B), the small plantlets were taken to growth cabinet for acclimatization, and maintained for further observations under controlled conditions of light, temperature and humidity. Then the plantlets transferred to vermiculite pot filled with sterilized soil: cowdung (1:1) and soil mixture treated with a solution of 1% IBA and ultimately transferred to shade house for acclimatization. In the shade house, the top of the pots were covered with transparent plastic sheet and grew at room temperature for 14 days with periodic irrigation (2 days interval). Thus in the growth cabinet and in the shade house, plants are acclimatized and hardened

before being transferred to the field conditions. The results of acclimatization or "hardening-off' have been presented in Table 8 and Plate 17-19.

Table 8. Survial rate of *in vitro* regenerated plants of *Stevia rebaudiana* B.

Acclimatization	No. of plants transplanted	Duration of Observation	No. of plants survived	Survival rate
In growth cabinet	20	7days	18	90
In shade house	20	14 days	16	80
In pot culture	20	30 days	16	80

It was revealed that 90% of plantlets were survived to growth cabinet (Table 8 and Plate 18 A). In shade house about 80% of the plantlets survived (Table 8; Plate 18 B). After being transferred to the pot (field condition), survival rate was 80% (Table 8 and Plate 19). It was also revealed that regenerated plants were morphologically similar to the mother plant. Regenerated plantlets of *Stevia* were successfully hardened and acclimatized in glass jar covered with poly propylene cap under net house conditions with 94.8 percent survival (Verma *et al.*, 2011).

17.(A) **17.(B)**

Plate 17: Plant ready for acclimatization. (A) directly generated plant (B) indirectly generated plant through callus

18. (A) 18. (B)

Plate 18. Acclimatization of regenerated plants (A) in growth cabinet (B) in shade house

Plate 19. Plant in pot

Chapter V
Summary And Conclusion

CHAPTER V

SUMMARY AND CONCLUSION

5.1 Summary

To look into the effect of different plant growth regulator on multi shoot proliferation both directly from explants as well as indirectly through callus induction and root formation along with acclimatization for *in vivo* survival, this experiment was conducted at Biotechnology Laboratory, Department of Biotechnology, Sher-e-Bangla Agricultural University, Sher-e-Bangla Nagar, Dhaka-1207, period of June, 2014 to August, 2015.

Shoot tips and nodal segments of 1-2cm length collected from healthy, disease free plants were used as explants for the study for *in vitro* regeneration. The major findings have been presented below.

For direct shoot induction applying BA or KIN alone maximum shoot induction (83.42%) was found in the treatment with 2.0 mg/L BA and the lowest percentage (50.98%) was induced in hormone free media in *S. rebaudiana*.

Minimum days were recorded to shoot induction (10.6) by the treatment of 2.0 mg/L BA where minimum (11) days was required by 1.0 mg/L KIN. The maximum days (18.4) to shoot induction were recorded in control.

The highest number of shoot (10.8) were recorded for 2.0 mg/L BA, and highest (10.2) number was recorded for 1.0 mg/L KIN. The lowest number of shoot (1.6) was found with hormone free media after 4 weeks of inoculation.

The maximum number of leaf per explants (17.20) was noticed from 2.0 mg/L BA, and (15.4) highest leaf number was recorded for 1.0 mg/L KIN, whereas the minimum (6.6) was recorded in control. The average length of shoot (3.72cm) was noticed from the 2.0 mg/L BA, and it was average (3.94cm) length for 1mg/L KIN. The minimum length (2.1) was found in control. The length of longest shoot (4.2 cm) was noticed from the 2mg/L BA, and it was longest (4.16 cm) for 1mg/L KIN.

For indirect shoot initiation through callus, callus was produced by the treatment BA+IBA and KIN+IBA. Between the two combination it was minimum (13.4) days for the treatment of 1.0 mg/L KIN + 1.5 mg/L IBA and minimum (14) days for 2.0 mg/L BA+ 1.5 mg/L IBA recorded for callus induction.

1.0 mg/L KIN + 1.5 mg/L IBA required minimum 11.4 days and 2.0 mg/L BA + 1.5 mg/L IBA required minimum 9.6 days were recorded for shoot induction from callus. The highest number of shoot (9.4) were recorded for 1.0 mg/L KIN + 1.5 mg/L IBA, highest (10.4) number was recorded for 2.00 mg/L BA + 1.5 mg/L IBA at 4WAI.

In case of callus induction potentiality, the highest induction potentiality (82.02% and 83.58% respectively) was observed with the treatment of 1.0 mg/L KIN +1.5 mg/L IBA and 2.0 mg/L BA +1.5 mg/L IBA.

For root induction directly produced shooted plants were cultured separately in media contained IBA. On the other hand media which produced callus containing rooting hormone eg. BA+IBA and KIN+IBA simultaneously with shoot also produced root.

In case of root induction potentiality, the highest induction potentiality (78.18% and 76.92% respectively) was observed with the treatment of 1mg/L IBA and 1.0 mg/L + 0.5 mg/L IBA. Minimum regeneration potentiality was recorded for media lacking growth regulator.

Minimum days (12.6) were recorded to root induction for the treatment of 1.0 mg/L IBA and minimum days (13.4) was required for 1.0 mg/L KIN + 0.5 mg/LIBA. The maximum days (25.2) to root induction were recorded in control.

The highest number of roots (7.2) were recorded for 1.0 mg/L IBA, highest (7.2) number was recorded in case of 1.0 mg/L KIN + 0.5 mg/L IBA, and the lowest number of root (2.4) was found with hormone free medium after 4 weeks of inoculation.

The average length of root (3.96cm) was noticed from 1.0 mg/L IBA, and it was average (3.64cm) length for 1.0 mg/L KIN + 0.5 mg/L IBA. The minimum length (1.32cm) was recorded in control. The length of longest root (4.18 cm) was noticed from the 1mg/L IBA, and it was longest (3.94 cm) for 1.0 mg/L KIN + 0.5 mg/L IBA at 4 WAI.

For acclimatization, plantlets were transferred from culture media to growth cabinet, where the highest survival rate (90%) was recorded. Then plantlets are transferred to shade house, outside of *in vitro,* where survival percentage was 80%. After hardening, plantlets were transferred to plastic pot i.e. field condition, where the survival rate of 80% was recorded.

5.2 Conclusion

Following conclusions can be made from the present study:

i. A micropropagation protocol has been developed in *Stevia.*

ii. Regenerated plants were found to be morphologically similar to the mother plant.

iii. For direct shoot regeneration single treatment of BA and KIN found better than combined treatments. The combination of KIN+IBA performed better than BA+KIN for indirect shoot regeneration through callus.

iv. For root regeneration IBA in low concentration performed better. The treatment 1.0 mg/L IBA showed best performance on root induction.

v. Findings of the present study showed that *in vitro* culture is effective method in the proliferation of *Stevia* and this experiment can be a useful tool for micropropagation of *Stevia.*

Chapter VI
Recomendations

CHAPTER VI

RECOMMANDATIONS

Following recommendations could be addressed based on the present experiment:

i. In the present experiment very few types and levels of hormone have been used which can be extended for *in vitro* regeneration of *Stevia* to identify further better combination if any.

ii. In addition to shoot tip and nodal segments, meristem and root tip culture can be practices for virus free plantlet regeneration.

iii. For callus induction many other combinations also may be tried.

iv. Influence of other factors (elicitors, antioxidants) such as ascorbic acid, activated charcoal, thidiazuron (TDZ), $CuSO_4$ (Copper sulfate) etc. should be examined.

v. Biochemical analysis for the active ingredient of *Stevia* may be tried.

Chapter VII
References

CHAPTER VII

REFERENCES

Abd Alhady, M. R. A. (2011). Micropropagation of *Stevia rebaudiana* Bertoni. A New Sweetening Crop in Egypt. *Global J. of Biot. & Bioch.* **6** (4): 178-182.

Ali, A., Gull, I., Naz, S. and Afgan, S. (2010). Biochemical investigation during different stages of *in vitro* propagation of *Stevia rebaudiana*. *Pak. J. Bot.* **42**(4): 2827-2837.

Ali, G., Fazal, H., Zahir, A., Muhammad, T., Muhamad, A. K. (2007). Callus induction and in vitro complete plant propagation of different cultivators of Tobacco (*Nicotina tabacum* L.) On media different hormonal concentration, Chakdara, Pakistan.

Asamenew, M. T. and Narayanaswamy, P. (2000). Induction of callus and plant regeneration in Coleus forskohlii Briq. *J. Appl. Hort.* **2**: 28-30.

Baskaran, P. and Jayabalan, N. (2005). An efficient micropropagation system for *Eclipta alba*, a valuable medicinal herb. *In vitro Cellular and Developmental Biol. Plant.* **41**: 532–539.

Brandle, J. E. and Rosa, N. (1992). Heritability of yield, leaf-stem ratio and stevioside content estimated from a ladrace cultivar of *Stevia*. *Canadian J. of Plant Sci.* **72**(4): 1263-1266.

Chotikadachanarong, K. and Dheeranupattana, S. (2013). Micropropagation and Acclimatization of *Stevia rebaudiana* Bertoni. *Pak. J. of Bio. Sc.* **16**(17): *887-890.*

Chun-Lai, Z. *et al.* (2004). Efficient procedures for callus induction and adventitious shoot organogenesis in sugar beet (*Beta vulgaris* L.) breeding lines. *In vitro* cellular & developmental biology. *Plant* . **40**(5): 475-481.

Das, A., Gantait, S. and Mandal, N. (2011). Micropropagation of an Elite Medicinal Plant: *Stevia rebaudiana* Bert. *Intl. J. of Agril* Res. **6**: *40-48.*

Debnath, M. (2008). Clonal propagation and antimicrobial activity of an endemic medicinal plant *Stevia rebaudiana*. *J. of Med. Plant. Res.* **2**(2): 045-051

Deshmukh, S. and Ade, R. (2012). In vitro rapid multiplication of *Stevia rebaudiana*: an important natural sweetener herb. *Nusantara Biosci.* **4**: 105-108.

Ghauri, E. G., Afridi, M.S., Marwat, G.A., Rahman, I. and Akram, M.(2013). Micro-Propagation of *Stevia Rebaudiana* Bertoni Through Root Explants. *Pak. J. Bot.* **45**(4): 1411-1416.

Giridhar, P., Sowmya, K. S., Ramakrishna, A. and Ravishankar, G. A. (2010). Rapid Clonal Propagation of Stevioside Profiles of *Stevia rebaudiana* Bertoni. *Intl. J. of plant devl. biol.* **4**(1): 47-52.

Gupta, P., Sharma, S. and Saxena, S. (2010). Callusing in *Stevia rebaudiana* (Natural Sweetener) for Steviol Glycoside Production. *Intl. J. of Biol., Biomol., Agril., Food and Biotech. Eng.* **4**(12).

Hossain, M. A., Kabir, S. A. H. M., Jahan, T. A. and Hasan, M. N. (2008). Micropopagation of Stevia. *Int. J. Sustain. Crop Prod.* **3**(3): 1-9.

Huxley, A. (1992). The New RHS Dictionary of Gardening. *Mac Millan Press.*, ISBN 0-333-47494-5.

Hwang, S. J. (2006). Rapid *in vitro* propagation and enhanced stevioside accumulation in *Stevia rebaudiana* Bert. *J. Plant Biol.* **49**(4): 267-270.

Ibrahim, I. A., Nasr, M.I., Mohammed, B. R. and Zefzafi, M. M. E. L. (2008). Nutrient Factors Affecting *In vitro* Cultivation of *Stevia rebaudiana. Sugar tech.* **10**(3): 248-253.

Jagatheeswari, D. and Ranganathan, P. (2012). Studies on Micropropagation of *Stevia rebaudiana Bert. Int. J. of Pharml. & Biol. Archives.* **3**(2): 315-320.

Janarthanam, B., Gopalakrishnan, M., Lakshmi Sai, G. and Sekar, T. (2009). Plant Regeneration From Leaf Derived Callus of *Stevia rebaudiana* Bertoni. *Plant Tiss. Cult. & Biotec.* **19**(2): 133-141.

Jitendra, M., Monika, S., Ratan, S. D., Priyanka, G., Priyanka, S. and Kiran, D. J. (2012). Micropropagation of an Anti diabetic Plant - *Stevia rebaudiana Bertoni*, (Natural Sweetener) in Hadoti Region of South-East Rajasthan, India. *J. of Biol. Sci.* **1**(3): 37-42.

Kalpana, M., Anbazhagan, M., Natarajan , V. (2009). Utilization of liquid medium for rapid micropropagation of *Stevia rebaudiana* Bertoni. *J. of Ecobiot.* **1**(1): 016-020.

Karim, M.A. , Jannat, R., Rahman, M. S. and Haque, M. S. (2008). Micropropagation Of *Stevia* Plant From Nodal Segments. *Progress. Agric.* **19**(2): 21-26

Lata, H., Chandra, S., Wang, Y.H., Raman, V., and Kha, E.A. (2013). TDZ-Induced High Frequency Plant Regeneration through Direct Shoot Organogenesis in *Stevia rebaudiana* Bertoni: An Important Medicinal Plant and a Natural Sweetener. *American J. Plant Sci.* **4**: 117-128.

Maina, S. M., Quinata, E., Kiran, K., Sharma, K., Simon, T., Moses, G. and Santie, M. D. V. (2010). Surface sterilant effect on the regeneration efficiency from cotyledon explant of groundnut (*Arachis hypogea* L) varieties adapted to eastern and Southern Africa. *J. Biotech.* **9**(20): 2866- 2871.

Moktaduzzaman, M., and Rahman, S. M. M. (2009). Regeneration of *Stevia rebaudiana* and analysis of somaclonal variation by RAPD. *Biotech.* **8**: 449–455.

Namdari, N., Shooshtari, L. and Qaderi, A. (2015). *In vitro* Micropropagation of *Stevia rebaudiana* Bertoni. *Biol. Forum – An Int. J.* **7**(1): 1750-1754.

Noordin, N., Ibrahim, R., Sajahan, N. H., Nahar, S. M. M., Nahar, S. H. M. and Rashid, N. R. A. (2012). Micropropagation Of *Stevia rebaudiana* Bertoni Through Temporary Immersion Bioreactor System. *Applied Life Sci.* **60**: 8.

Pasha, F. (2014). *In vitro* Micropropagation and Media Optimization Of *Stevia rebaudiana* an important bio-sweetener and medicinal plant. *Int. J. Applied Bio. And pharmacy. Tech.* **5**(1): 12-16.

Pawar, S. V., Khandagale, V. G., Jambhale, V. M., Jadhav, A. S. and Pawar, B. D. (2015). *In vitro* Regeneration Studies In *Stevia* Through Nodal Segment And Shoot Tip. *The Biosci.* **10**(3): 1007-1010.

Preethi, D., Sridhar, T. M. and Naidu, C. V. (2011). Tissue Culture Direct Shoot Organogenesis from Leaf Explants of *Stevia rebaudiana*. *J. of Phytol.* **3**(5): 69-73.

Puri, M., Sharm, D. and Tiwari, A.K. (2011). Downstream processing of stevioside and its potential applications. *Biot. Advances.* **29**(6): 781-791.

Rafiq, M., Dahot, M. U., Mangrio, S. M., Naqvi, H. A. and Qarshi, I. A., (2007). *In vitro* clonal propagation and biochemical analysis of field established *Stevia rebaudiana* Bertoni. *Pak. J. Bot.* **39**(7): *2467*-2474.

Ramesh, K., Singh, V. and Megeji, N. W. (2006). Cultivation of Stevia [*Stevia rebaudiana* (Bert.)Bertoni]: a comprehensive review. *Adv. in Agron.* **89**: 137-177.

Rao, S. P. N. (2008). The role of endogenous auxin in root initiation. *Plant Growth Regul.* **13**(1): 77-84.

Razak, U. N. A. A., Ong, C. B., Yu, T. S. and Lau, L. K. (2014). *In vitro* micropropagation of *Stevia rebaudiana* Bertoni in Malaysia. *Braz. Arch. Biol. Tech.* **57**(1): 1516-8913.

Russell, L. M. (1979). Regeneration of whole plants from callus culture of diverse genetic lines of *Pisum sativum. L. Planta.* **146**: 243- 244.

Shende, C. B. and Manik, S. R. (2013). Direct regeneration from axillary bud explants of *Stevia rebaudiana* Bertoni- A medicinal plant. *J. of Agril. Techn.* **9**(7): 2005-2011.

Sikdar, S. U., Zobayer, N., Azim, F., Ashrafuzzaman, M. and Prodhan, S. H. (2012). An efficient callus initiation and direct regeneration of *Stevia rebaudiana. African J. of Biot.* **11**(45): 10381-10387.

Singh, P. and Dwivedi, P. (2013). Efficient Micropropagation Protocols of Regeneration of Stevia rebaudianaBertoni, An anti-diabetic Herb. *Soc. For Plant Res.* **26** (1): 318-323

Sivaram, L. and Mukhundan, U. (2003). In vitro culture studies on *Stevia rebaudiana. In vitro* cellular and developmental biology. *Plant.* **39**: 520-523.

Slavova, Y., Nenkova, D. and Ivanova, I. (2003). Study on the influence of the substance of benzymidazol upon *Stevia rebaudiana* Bertoni cultivated *in vitro. Bulg. J. Agric. Sci.* **9**: 225-228.

Soejarto, D. D. *et al.* (1982). Potenitial sweetening agents of plants origin. *J. Nat. Prod.* **45**: 590-599.

Soliman, H. I. A., Metwali, E. M. R., Almaghrabi, O. A. H. (2014). Micropropagation of Stevia rebaudiana Betroni and assessment of genetic stability of *in vitro* regenerated plants using inter simple sequence repeat (ISSR) marker. *Plant Biotech.* **31**: 249–256.

Srivastava, N., Barkha, K., Vikas, S., Yogesh, K. N., Dobriyal, A. K., Sanjay, G. and Vikash, S.J. (2010). Standardization of sterilization protocol for micropropagation of *Aconitum heterophyllum*-An endangered medicinal herb. *Acad. Arena.* **2**(6): 37-42.

Sumaryono and Sinta, M. M. (2011). Increasing shoot multiplication rate and plantlet vigor of *Stevia rebaudiana in vitro* culture. Menara Perkebunan. **79**(2): 49-56.

Swanson, S. M., Mahady, G. B. and Christopher, W. W. B. (1992). Stevioside biosynthesis by callus, root, shoot and rooted-shoot cultures *In vitro. Plt. Cell Tiss. and Org. Cult.* **28**: 151-157.

Tadhani, M. B., Jadeja, R. P. and Rena, S. (2006). Micropopagation of *Stevia rebaudiana* Bertoni using multiple shoot culture. *J. Cell Tiss. Res.* **6**: 545-548

Taleie, N., Hamidoghli, S. and Hamidoghli, Y. (2012). *In vitro* Plantlet Propagation Of *Stevia rebaudiana* Bertoni. *South West J. Hortic. Biol. Environ.* **3**(1): 99-108

Thiyagarajan, M. and Venkatachalam, P. (2012). Large scale *in vitro* propagation of *Stevia rebaudiana* (Bert.) for commercial application. *Pharmaceutically important and antidiabetic medicinal herb.* pp: 111–117.

Tiwari , S., Arnold, R., Saxena, A., Mishra , R. M., Tiwari , A.S., Rajak, A., and Singh, P.(2013). Studies on rapid micropropagation of *Stevia rebaudiana Bertoni*: A natural sweetener. *Int. J. of Pharm. & Life Sci.* **4**(5): 2667-2671.

Trauttman, I. A. and Visser, J. H. (1990). An *in vitro* study of organogenesis in guayule (*P. argentatum*). *Plant Sci.* **72**: 275-281.

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

Vazquez-Baxcajay, Robledo-Paz, Alejandrina, Muratalla-Lua, Alfonso, Y., Conde-Martinez and Víctor. (2014) Micropropagation of *Stevia rebaudiana* Bertoni, and Steviosides Detection. *Bioagro [Online].* **26**(1): 49-56.

Verma, S., Yadav, K. and Singh, N. (2011). Optimization of the Protocols for Surface Sterilization, Regeneration and Acclimatization of Stevia rebaudiana Bertoni. *American-Eurasian J. Agric. & Environ. Sci.* **11** (2): 221-227.

Yadav, N. (2016). Conservation of some endangered and economically important medicinal plants of India. *J. Integr. Sci. Tech.* **4**(2): 59-62.

Yamazaki, T. and Flores, H.E. (1991). Examination of steviol glucosides production by hairy root and shoot cultures of *Stevia rebaudiana*. *J. Natl. Prods.* **54**(4): 986-992.

Yarra, R., Aileni, M., Vemunoori, A. K., Kokkirala, V. R., Umate, P. and Abbagani, S. (2010). Direct Shoot Regeneration From Mature Leaf Explants Of *Sphaeranthus indicus* L., A Multipurpose Medicinal Plant. *J. of Phytol.* **2**(5): 05–11.

Chapter VIII
Appendices

CHAPTER VIII

APPENDICES

Appendix 1. Composition and concentrations used for the preparation of MS medium (Murashige and Skoog, 1962).

Components	Concentration(mg/L)
Macronutrients	
KNO_3	1900
NH_4NO_3	1650
$MgSO_4.7H_2O$	370
$CaCl_2.2H_2O$	440
KH_2PO_4	170
Micronutrients	
$MnSO_4.4H_2O$	22.3
H_3BO_3	6.2
$ZnSO_4.7H_2O$	8.6
$CuSO_4.5H_2O$	0.025
$Na_2MoO_4.2H_2O$	0.25
$CoCl_2.6H_2O$	0.025
KI	0.83
Iron Source	
$Fe_2SO_4.7H_2O$	27.8
Na_2-EDTA	37.3
Organic nutrients	
Nicotinic acid	0.5
Pyridoxine HCl	0.5
ThaimineHCl	0.1
Glycine	2.0
Myo-inositol	100
Sucrose	3000.00
Agar	7000.00

Appendix 2. Analysis of variance on days to shoot induction

Source of Degrees of Freedom	Degrees of Freedom	Sum of Squares	Mean Square	F-value	Probability
Treatment	10	240.909	24.0909	17.91	0.0000
Error	44	59.200	1.345		
Total	54	300.109			
CV (%)	8.70				
$LSD_{(0.05)}$	1.4785				

Appendix 3. Analysis of variance on no. of shoots per explants at 2 WAI

Source of Degrees of Freedom	Degrees of Freedom	Sum of Squares	Mean Square	F-value	Probability
Treatment	10	23.200	2.3200	4.73	0.0001
Error	44	21.600	0.49091		
Total	54	44.800			
CV (%)	31.85				
$LSD_{(0.05)}$	0.8931				

Appendix 4. Analysis of variance on no. of shoots per explants at 4 WAI

Source of Degrees of Freedom	Degrees of Freedom	Sum of Squares	Mean Square	F-value	Probability
Treatment	10	342.400	34.2400	32.19	0.0000
Error	44	46.800	1.0636		
Total	54	389.200			
CV (%)	13.94				
$LSD_{(0.05)}$	1.3146				

Appendix 5. Analysis of variance on Average length of shoot

Source of Degrees of Freedom	Degrees of Freedom	Sum of Squares	Mean Square	F-value	Probability
Treatment	10	11.4698	1.14698	40.05	0.0000
Error	44	1.2600	0.02864		
Total	54	12.7298			
CV (%)	5.03				
$LSD_{(0.05)}$	0.2344				

Appendix 6. Analysis of variance on length of longest shoot

Source of Degrees of Freedom	Degrees of Freedom	Sum of Squares	Mean Square	F-value	Probability
Treatment	10	12.0840	1.20840	35.73	0.0000
Error	44	1.4880	0.03382		
Total	54	13.5720			
CV (%)	5.05				
$LSD_{(0.05)}$	1.5033				

Appendix 7. Analysis of variance on the number of leaves per explant

Source of Degrees of Freedom	Degrees of Freedom	Sum of Squares	Mean Square	F-value	Probability
Treatment	10	368.327	36.8327	26.48	0.0000
Error	44	61.200	1.3909		
Total	54	429.527			
CV (%)	8.96				
$LSD_{(0.05)}$	0.9940				

Appendix 8. Analysis of variance on the percentage of explants showing shoot induction

Source of Degrees of Freedom	Degrees of Freedom	Sum of Squares	Mean Square	F-value	Probability
Treatment	10	3755.17	375.517	617.44	0.0000
Error	44	26.76	0.608		
Total	54	3781.93			
CV (%)	1.05				
$LSD_{(0.05)}$	0.9940				

Appendix 9. Analysis of variance on Days to callus induction

Source of Degrees of Freedom	Degrees of Freedom	Sum of Squares	Mean Square	F-value	Probability
Treatment	7	33.7750	4.82500	8.04	0.0000
Error	32	19.2000	0.600		
Total	39	52.9750			
CV (%)	5.17				
$LSD_{(0.05)}$	0.9979				

Appendix 10. Analysis of variance on Days for shoot induction from callus

Source of Degrees of Freedom	Degrees of Freedom	Sum of Squares	Mean Square	F-value	Probability
Treatment	7	98.300	14.0429	20.80	0.0000
Error	32	21.600	0.6750		
Total	39	119.900			
CV (%)	6.60				
$LSD_{(0.05)}$	1.0584				

Appendix 11. Analysis of variance on No. of shoots per callus 2 WAI

Source of Degrees of Freedom	Degrees of Freedom	Sum of Squares	Mean Square	F-value	Probability
Treatment	7	8.000	1.14286	3.52	0.0000
Error	32	10.400	0.32500		
Total	39	18.400			
CV (%)	25.91				
$LSD_{(0.05)}$	0.7344				

Appendix 12.Analysis of variance on No. of shoots per callus 4 WAI

Source of Degrees of Freedom	Degrees of Freedom	Sum of Squares	Mean Square	F-value	Probability
Treatment	7	49.600	7.08571	10.90	0.0000
Error	32	20.800	0.6500		
Total	39	70.400			
CV (%)	9.71				
$LSD_{(0.05)}$	1.0386				

Appendix 13.Analysis of variance on the percentage of explants showing callus induction

Source of Degrees of Freedom	Degrees of Freedom	Sum of Squares	Mean Square	F-value	Probability
Treatment	7	134.875	19.2679	15.50	0.0000
Error	32	39.784	1.2433		
Total	39	174.659			
CV (%)	1.39				
$LSD_{(0.05)}$	1.4364				

Appendix 14.Analysis of variance on Days for root induction

Source of Degrees of Freedom	Degrees of Freedom	Sum of Squares	Mean Square	F-value	Probability
Treatment	12	546.585	45.5487	68.85	0.0000
Error	52	34.400	0.6615		
Total	64	580.985			
CV (%)	5.09				
$LSD_{(0.05)}$	1.0322				

Appendix 15. Analysis of variance on No. of roots per explants 2 WAI

Source of Degrees of Freedom	Degrees of Freedom	Sum of Squares	Mean Square	F-value	Probability
Treatment	12	30.1538	2.51282	6.41	0.0000
Error	52	20.4000	0.39231		
Total	64	50.5538			
CV (%)	23.53				
$LSD_{(0.05)}$	0.7949				

Appendix 16. Analysis of variance on No. of roots per explants 4 WAI

Source of Degrees of Freedom	Degrees of Freedom	Sum of Squares	Mean Square	F-value	Probability
Treatment	12	87.354	7.27949	8.84	0.0000
Error	52	42.800	0.82308		
Total	64	130.154			
CV (%)	16.38				
$LSD_{(0.05)}$	0.1514				

Appendix 17. Analysis of variance on Average length of roots per explants 4 WAI

Source of Degrees of Freedom	Degrees of Freedom	Sum of Squares	Mean Square	F-value	Probability
Treatment	12	22.6018	1.88349	130.94	0.0000
Error	52	0.7480	0.01438		
Total	64	23.3498			
CV (%)	3.72				
$LSD_{(0.05)}$	0.1522				

Appendix 18. Analysis of variance on length of longest roots per explants 4 WAI

Source of Degrees of Freedom	Degrees of Freedom	Sum of Squares	Mean Square	F-value	Probability
Treatment	12	22.9025	1.90854	617.44	0.0000
Error	52	0.5320	0.01023		
Total	64	23.4345			
CV (%)	2.90				
$LSD_{(0.05)}$	0.1284				

Appendix 19. Analysis of variance on the percentage of explants showing root induction

Source of Degrees of Freedom	Degrees of Freedom	Sum of Squares	Mean Square	F-value	Probability
Treatment	12	13211.1	1100.92	1454.32	0.0000
Error	52	39.4	0.76		
Total	64	13250.4			
CV (%)	1.24				
$LSD_{(0.05)}$	1.1042				