IN VITRO PLANTLET REGENERATION IN MINT (*Mentha spicata*)

SAYED SHAQUR AHMED

Reg. No.: 12-05158

DEPARTMENT OF BIOTECHNOLOGY
SHER-E-BANGLA AGRICULTURAL UNIVERSITY
DHAKA-1207

June, 2018

IN VITRO PLANTLET REGENERATION IN MINT (*Mentha spicata*)

BY

SAYED SHAQUR AHMED

Reg. No.: 12-05158

A Thesis

Submitted to the Department of Biotechnology, Faculty of Agriculture
Sher-e-Bangla Agricultural University, Dhaka-1207,
In partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE (MS)

IN

BIOTECHNOLOGY

SEMESTER: JANUARY- JUNE, 2018

APPROVED BY

Fahima Khatun Assistant Professor Department of Biotechnology Sher-e- Bangla Agricultural University Dhaka-1207 **Supervisor**	**Homayra Huq** Associate Professor Department of Biotechnology Sher-e- Bangla Agricultural University Dhaka-1207 **Co-Supervisor**

Prof. Dr. Md. Ekramul Hoque
Chairman
Examination Committee
Department of Biotechnology
Sher-e- Bangla Agricultural University
Dhaka-1207

DEPARTMENT OF BIOTECHNOLOGY
Sher-e- Bangla Agricultural University
Sher-e-Bangla Nagar, Dhaka-1207

CERTIFICATE

*This is to certify that thesis entitled, "**IN VITRO** PLANTLET REGENERATION OF MINT **(Mentha spicata)"** submitted to the Department of Biotechnology, Sher-e-Bangla Agricultural University, Dhaka, in partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE (MS) in BIOTECHNOLOGY**, embodies the result of a piece of bona fide research work carried out by **SAYED SHAQUR AHMED** Registration No. **12-05158** 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.

Dhaka, Bangladesh

Fahima Khatun
Assistant Professor and Supervisor
Department of Biotechnology
Sher-e- Bangla Agricultural University
Dhaka-1207

DEDICATED TO

My beloved parent

ACKNOWLEDGEMENTS

The author would like to express his deepest sense of gratitude, endless praises and thanks to the Almighty Allah who enabled him kind blessing to complete this piece of research work and to submit the dissertation.

The author would like to express his deepest sense of gratitude and profound respect to his research supervisor **Fahima Khatun,** Assistant professor, Department of Biotechnology, Sher-e-Bangla Agricultural University (SAU). Dhaka-1207, for her constant supervision, valuable suggestion, scholastic guidance, continuous inspiration, constructive comments and immense help in conducting the research work and preparation of the dissertation.

The author expresses his sincere appreciation, respect and immense indebtedness to the respected members of co-supervisor, **Homayra Huq,** Associate professor, Department of Biotechnology, Sher-e-Bangla Agricultural University (SAU). Dhaka-1207, for her cordial suggestions, constructive criticisms and valuable advice during research work.

The author expresses his heartfelt gratefulness to **Prof. Dr. Md. Ekramul Hoque**, Department of Biotechnology, Sher-e-Bangla Agricultural University (SAU), Dhaka-1207, for his inspiration and encouragement.

The author also his heartfelt gratitude to the members of the family for his blessing, encouragement and constant inspiration throughout his academic career. Thanks to all of his nearest and friends for their help and good wishes all the time.

June, 2018

SAU, Dhaka **The Author**

***IN VITRO* PLANTLET REGENERATION IN MINT (*Mentha spicata*)**

ABSTRACT

The present study was undertaken in Biotechnology Laboratory of the Department of Biotechnology, Sher-e-Bangla Agricultural University, Sher-e-Bangla Nagar, Dhaka-1207, during the period from June, 2017 to January, 2018 to evaluate the effect of different plant growth regulators (BA, IBA) on *in vitro* plantlet regeneration in Mint. The shoot tips of Mint were used as explants which were inoculated in MS media supplemented with different conc. (1.0, 1.5, 2.0 and 2.5 mg/l) of Benzyl adenine (BA) and Indole-3-butyric acid (IBA) (0.5, 1.0, 1.5 and 2.0 mg/l) either alone or in combination of both. The experiments were arranged in Completely Randomized Design (CRD) with three replications. The maximum shoot induction was obtained with 2.0 mg/L BA. The highest shoot length (12.12 cm) was found in 2.0 mg/L BA and minimum shoot length (2.90 cm) in control treatment. The highest percentage (85.00%) of root induction was recorded with BA 2.0 mg/l + IBA 1.0 mg/l in minimum (11.0 days). The highest length of root (3.25, 5.00 and 7.50 cm) at 21, 28 and 42 DAI respectively was found in 1.0 mg/l IBA and the control treatment found the lowest length of root at all DAI. The treatment BA 2.0 mg/l + IBA 1.0 mg/l gave the highest number of root (10, 15 and 20.67) at 21, 28 and 42 DAI respectively. Regenerated plantlets showed 90% survival during in growth chamber and 83.33% in shade house and 80 % in open atmosphere at direct sunlight. Therefore, an efficient protocol has been developed for *in vitro* regeneration of Mint which has great commercial value for year round production in Bangladesh.

LIST OF CONTENTS

LIST OF CONTENTS (cont'd)

LIST OF CONTENTS (cont'd)

LIST OF CONTENTS (cont'd)

LIST OF CONTENTS (cont'd)

LIST OF TABLES

LIST OF FIGURES

LIST OF PALATES

LIST OF ABBREVIATED TERMS

Abbreviation		Full Word
Agril.	:	Agricultural
Biol.	:	Biological
BAP	:	6- Benzyl Amino Purine
BA	:	Benzyl adenine
BARI	:	Bangladesh Agricultural Research Institute
cm	:	Centimeter
CRD	:	Completely Randomize Design
cv.	:	Cultivar
Conc.	:	Concentration
2, 4-D	:	2,4- Dichlorophenoxy acetic acid
WAI	:	Weeks After Induction
Dw	:	Distilled water
DMRT	:	Duncan's Multiple Range Test
et. al.	:	And others
FAO	:	Food and Agriculture Organization
g/L	:	Gram per litre
IAA	:	Indole acetic acid
IBA	:	Indole butyric acid
NAA	:	α-Napthalene acetic acid
Int.	:	International
J.	:	Journal
Kin	:	kinetin
Mol.	:	Moleculer
mg/L	:	Milligram per litre
μM	:	Micromole
MS	:	Murashige and Skoog
PGRs	:	Plant Growth Regulators
Res.	:	Research
Sci.	:	Science
CV	:	Co-efficient of Variation
^{o}C	:	Degree Celsius
etc.	:	Et cetera

CHAPTER I

INTRODUCTION

Mint (*Mentha spp*.) is a perennial plant belongs to the family Lamiaceae and has approximately 25 species. It is an aromatic plant that contains volatile essential oils, used fresh or dried as flavoring agent in a wide variety of foods. Mint oils are used in making different dental and medicinal products as well as in cosmetic industries. It is commonly known as "Pudina" having dark green leaves and is usually found near ponds and other humid places of homestead (Bhat *et al*., 2002). Mint cultivation is widely distributed around the world. The United States of America is the main producer of peppermint and spearmint followed by India (Anon, 2008).

Peppermint and spearmint are the most commercially exploited species of mint because they have anti-feeding, insecticidal, anti-viral, anti-bacterial, immuno modulating and anti-aging properties (Ali *et al*., 2002). Mint leaves are used in teas, beverages, jellies, syrups, candies and ice creams.

In Bangladesh, mint is grown scatterdly all over the country, but there is no statistics available in our country about area and production of mint. Presently, mint oil is imported from abroad for using in industries (Hoque, 2013). The highest fresh herb yield (12.83 t/ha) of Mint was obtained from proper irrigation and nitrogenous fertilizer application.

Mints are mainly propagated vegetatively rather than by seeds. The best way to propagate mints is by taking cuttings from superior species. Mint prefers fertile soil with a pH from 6.0 to 7.0. Frequent harvesting is the key to keeping mint plants at their best. Only some mints, such as *M. arvensis* L., *M. pulegonium* L. and *M. spicata* L., are propagated by seed (Heidari*et al*., 2012). This conventional process of vegetative propagation through stolon is a slow process and they are susceptible to many diseases (Safaeikhorram*et al*., 2008). In our climatic conditions, poor overwintering may occasionally produce an

insufficient number of seedlings. To overcome these problem *In vitro* propagation of mint is the best technique. It also offers year-round production, precise crop production scheduling and reduce stock plant space of crops. The main advantage of *in vitro* propagation lies not only in the mass scale production but also in the production of high quality and uniform planting material that can be multiplied on a year-round basis under disease-free conditions anywhere irrespective of the season and weather (Hoque, 2013).

Plant tissue culture is a modern technique mainly rooted on plant cell culture. This technique has been used in the multiplication of "good clones" of agricultural crops and medicinal plants. Plant cell culture is considered as an effective system for the study of the biological importance of bioactive metabolites in *in vitro* (Yanpaisan*et al*., 1999). The use of *in vitro* culture techniques can ensure speedy multiplication of valuable varieties (Sunandakumari*et al*., 2004) and the possibility to obtain biological material free from pathogens in large-scale (Kane, 2014).

A balance between auxin and cytokinin determines the *in vitro* regeneration of plants grown in artificial medium. Generally, cytokinin helps in shoot proliferation and auxin helps in callus formation and rooting of proliferated shoots. The presence of auxin in defined combinations with cytokinins in the culture medium is also necessary to obtain adventitious shoot formation (Caboni and Tonelli, 2009). However, the requirement of cytokinin and auxin depends on the plant species, genotype, explant type and culture conditions. Requirement of growth regulators depends on type of explants and its physiological condition also. The best media with optimum growth regulator, growth condition and suitable explants are needed to be standardized for large scale.

As an important medicinal plant, mint may be a source of income for domestic use as well as for export. Therefore, attempt was made to develop protocols for

large-scale *in vitro* propagation of locally available Mint (*Mentha spicata).* Considering the above facts, the present investigation has been undertaken to find out the performance of different hormones with the optimum concentration of BA and IBA for *in vitro* plantlets regeneration of Mint. The experiment was designed to fulfill the following objectives.

Objectives:

i. Establishment of *in vitro* regeneration protocol of Mint.

ii. Assessment of hormonal effect for *in vitro* response of Mint.

iii. Identification of best hormonal concentration for *in vitro* regeneration of Mint.

iv. To study the rapid regeneration of Mint within a short period of time.

CHAPTER II

REVIEW OF LITERATURE

The present investigation involved *in vitro* micropropagation of *Mentha spicata*. Plant regeneration from culture media of leaf explants followed by genetic stability of plantlets seems to be meager. However, information available in this aspects have been reviewed and presented in this sections:

2.1 *In vitro* regeneration of mint

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 shoot and root formation (Ali *et al*., 2007).

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. Mint is one of them. Many plant breeders of different countries as well as Bangladesh have been employing biotechnological tools for the development of Mint, 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.

2.2. Sterilization of explant

Both NaOCl and $HgCl_2$ are oxidizing agents and damage the microorganism by oxidizing the enzymes. 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% + 8HQC (200 ppm) 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. Multiple shoot induction

A simple micropropagation method from nodal and shoot tip explants is reported here for *Mentha viridis*, an economically important medicinal plant. High frequency of microshoots was obtained from these two explants on MS supplemented with various concentrations of BAP (1.0 - 4.0 mg/l) and Kn (1.0 - 4.0 mg/l). Maximum number of shoots was obtained from nodal explants in the medium containing 3.0 mg/l BAP. The root induction was carried out by using IBA and IAA (0.5 - 2.0 mg/l). Among these, high frequency of root proliferation was achieved in the medium containing 1.5 mg/l of IBA. The rooted plantlets were hardened and transferred to the field. The survival rate was 90 - 95% after 25 days (Hassan and Roy, 2005).

Zhang *et al*. (2005) developed a protocol for high frequency somatic embryogenesis and plant regeneration from cotyledon and hypocotyl explants of Mint somatic embryos developed into mature embryos on MS medium in the presence of 45 g 1- polyethylene glycol. After desiccation, somatic embryos developed into plantlets by culturing the mature somatic embryos on 1/2 x MS medium containing 0.24 pM indole-3-butyric acid (IBA).

Haque *et al*. (2016) showed that the propagation rate of Mint was significantly increased when IBA was added in a low concentration along with the optimum concentration of BA where BA along with IBA proved to be the best PGR combination for micropropagation.

Kehie *et al*. (2012) conducted that an efficient micro propagation protocol developed for the production of disease-free, cost-effective hybrid Mint plants thorough *in vitro* plant regeneration using different explants including root, hypocotyl, cotyledon, and shoot tips.

Rizwan *et al*. (2013) reported that the combinations of BA + IBA and BA alone induced shoot regeneration and higher shoot induction of Mint in nodes and shoot tip explants on MS-medium supplemented with lower dose of BA 2.5 mg/l in MS medium BA mg/l alone and BA 2.5 mg/l with IBA 1.0 mg/l promoted shoot induction.

Arous *et al*. (2001) conducted an experiment that the regeneration of Mint variety from zygotic embryos cultured *in vitro* condition from adventitious buds. The best results for bud induction were obtained in Murashige and Skoog medium, supplemented with 6-benzyl-aminopurine (3 mg/l) and naphthalene-acetic acid (1 mg/l). Important effect of 6-benzylaminopurine in adventitious bud formation was demonstrated. Shoot bud development was enhanced by the addition of gibberellic acid to the medium.

Raj *et al*. (2015) conducted that the regeneration of in multiple shoot was initially induced using MS medium supplemented with 2.5 mg/L BA and 1.0 mg/L IBA or MS medium fortified with BA alone or in combination with IBA and adenine sulphate.

Gayathri *et al*. (2015) developed an *in vitro* micropropagation protocol for *Mentha sp.* popularly known as peppermint. The study revealed that MS medium supplemented with BA alone or in combination with IBA, Adenine Sulphate for multiple shoot development. Maximum numbers of shoot buds were produced in MS medium containing at BA 2.0 mg/l.

Hasnat *et al.* (2007) established an efficient and reliable *in vitro* protocol for induction and regeneration of Mint. Better performance in both varieties regarding shoot initiation, regeneration rate (%) and number of lateral shoots per regenerants were achieved on medium containing 3.0 mg/l BA. Rooting was achieved on half strength MS basal medium containing 1.0 mg/l IBA.

Auer *et al.* (2002) showed that 3.0 mg/l BA was the most suitable concentration for regeneration (41-49%) in both varieties. This response could be attributed to the BA uptake and metabolism which was subsequently converted to isopently adenine (iP) and isopently adenosine (iPR) inhibiting the activity of cytokinin oxidase on cytokinin action in the early stages of shoot development.

Sanatmbi *et al*. (2007) established a novel micropropagation protocol for *Mentha sp* through induction of axillary shoot proliferation of *in vitro* raised plantlets by decapitation and using the axillary shoots as explants for multiple shoot bud induction. About 2–6 axillary shoots were induced within 2 weeks when 4-week-old *in vitro* raised plantlets were decapitated. The axillary shoot-tip explants produced multiple shoot buds when cultured on Murashige and Skoog's (MS) medium containing 8.8–44.4 mM 6-Benzyladenine or 9.3–46.7 mM kinetin alone or 8.8–44.4 mM BA with 4.6 mM kinetin or 5.7 and 28.5 mM indole-3-acetic acid (IAA). Maximum number of shoots (5.6) were induced on medium containing 22.2 mM BA in combination with 4.65 mM kinetin. The separated shoots rooted and elongated on medium containing 2.8 mM IAA or 2.4–4.9 mM indole-3-butyric acid (IBA).

Anilkumar (2010) conducted an experiment that the effectiveness of combination of 31 mM BAP and 4.6 mM kinetin for multiple shoot induction from shoot-tip explants in *Mentha sp.*

Christopher and Rajan (2010) reported that very high levels of BA (66.6–88.8 mM) and kinetin (92.9–116.2 mM) were necessary for maximal shoot proliferation from shoot tip explants of *Mentha sp.* However, in the present study, the number of shoot buds regenerated was reduced when the

concentration of BA was more than 22.2 mM or when the concentration of kinetin was more than 23.2 mM. When the regenerated shoot buds were separated and transferred to the rooting medium, rhizogenesis occurred followed by elongation of the shoot buds.

An experiment was conducted by Husain *et al*. (2007) found that various responses presented by the different types of Mint tested in this study confirms that *in vitro* Mint regeneration requirements depend on the cultivar.

Christopher *et al*. (2016) obtained that the multiple shoots from 10 per cent of cultured leaf segments of Mint on medium with 1 mg/l kinetin (kin) and 1 mg/l naphthalene acetic acid (NAA).

Regeneration from cotyledons of mature zygotic embryos was reported by Fari *et al*. (2003). A comparative study on *in vitro* regeneration of Mint leaf explants revealed that the best regeneration was from cotyledonary explant.

Arroya *et al*. (2016) found that percent regeneration of Mint was less in cotyledon explant compared to hypocotyl, found no significant differences among cotyledonary leaves, hypocotyls and leaf tissue with respect to frequency of shoots.

Ebida *et al*. (2002) observed proliferation of Mint buds from cut surfaces of cotyledon on 3 mg/l BA and 1.0 mg/l IBA. About 95 percent shoot regeneration from cotyledonary explant was obtained on MS medium supplemented with 5 mg/l BAP and 1.5 mg/l indole-3-buteric acid (IBA).

Kulkarni (2005) reported that number of multiple shoots induced was more in cotyledon compared to hypocotyl from explants of Mint on medium containing higher levels of BA.

Rama (2007) examined the morphogenetic response was good in cotyledonary leaves as compared to hypocotyls of stevia on MS medium with 2.5 mg/l BA and 1.0 mg/l IBA. Shoot buds were culturing them on a medium containing

combinations of BA with IBA. The shoot buds were rooted on a medium containing 0.5 mg/l IBA.

Mathew and Pious (2002) studied that whole cotyledons cultured on MS with 2 mg/l BAP + 1 mg/l IBA resulted in maximum average number of buds in Mint plant.

Satner *et al*. (2016) used shoot tip and axillary shoot explants for *in vitro* regeneration and mass multiplication of Mint. They reported that shoot tip explants excised from *in vitro* raised seedlings can be used for multiple shoot induction in MS medium supplemented with BAP alone or in combination with IAA. Maximum number of shoots were obtained on MS medium containing 22 mM BAP after four weeks of culture in the cultivar Haomorok.

Agawal *et al*. (2010) reported that shoot tip and hypocotyls segments of Mint were also used for producing shoots. Shoot tip explants cultured on MS medium supplemented with 5 mg/l BAP produced vigorous shoots and a large number of shoots, intervening callus was observed with 0 to 5 mg/l BAP and low levels of Indole Butyric acid (IBA).

Fari *et al*. (2003) observed shoots from apical segments of hypocotyl, while middle and basal segments produced roots and callus respectively. The best concentration for shoot differentiation from hypocotyls was 5 mg/l BAP.

2.4. Elongation of shoots

Ranjan *et al*. (2006) conducted that the efficient shoot induction has been observed but elongation of shoots into proper shoots is a consistent problem. Zhu *et al*. (2014) with a low concentration of BA or kinetin to assess its effect on shoot proliferation. They suggested that BAP might increase shoot proliferation by inhibiting the transport of auxins leading to a more favourable balance between cytokinins and auxins but they did not get good result with respect to elongation.

Szasz *et al*. (2005) studied 17 bell pepper genotypes for *in vitro* shoot forming capacity of Mint seedling explants and indicated that the induced buds developed but they usually formed pale, leaf like structures on 2.5 mg/l BA + 1.0 mg/l IBA.

Vikram *et al*. (2016) established that the increase the number of elongated shoots. In the first step, multiple buds obtained from cotyledonary leaves were cultured on MS + 2.0 mg/l BA + 0.5 mg/l IBA for 10 days and then transferred to MS + 1.0 mg/l IBA + 2.5 mg/l BA developed about 10 mature plants from a single explant.

A highly efficient three stage protocol for the regeneration of Mint from cotyledon explants was developed by Husain *et al*. (1999). This protocol used phenylacetic acid (PAA) in both the shoot induction medium and the medium for elongation of the shoots. A superior medium for the induction of buds from the cotyledons was MS medium supplemented with BAP (2 or 3 mg/l) + IBA (2 mg/l). Buds were elongated during the second stage on medium containing BA (2 to 5 mg/l) + IBA (2 mg/l). Bud elongation was achieved in 100 per cent of the cultures provided the buds were induced in the primary stage on a medium supplemented with BA + IBA. The shoots that elongated in the second stage rooted at 100 per cent frequency on a medium supplemented with IBA (1 mg/l).

2.5. Response of genotypes to shoot induction

High Frequency Rapid Plant Regeneration from Shoot Tip and Nodal Explants of *Mentha spicata* L. was described by Sujana *et al*. (2011). *Mentha spicata* L. (Spearrmint) is a perennial glabrous and strongly scented herb belonging to the family lamiaceae. The plant is aromatic, stimulant, and used for allaying nausea, headache and vomiting. Peppermint raw material is used in medicine, cosmetics and food industry, therefore this plant is widely grown around the world. Spearrmint produces a large amount of essential oils and has a good aroma, thus it is more widely grown, especially for industrial processing. An *in*

vitro regeneration system with a maximum efficiency rate was developed in *Mentha spicata* using shoot tip and nodal segments of three week old stock plants on Murashige and Skoog's medium supplemented with combination of 6- benzyl amino purine and naphthalene acetic acid (BAP - 1.5mg/L and NAA - 0.1 mg/L). Shoots developed at sites of excision directly from the cells. The highest numbers of shoots (53) were obtained on medium containing BAP and NAA. *In vitro* shoots were then excised from the shoot clumps and transferred to rooting medium containing indole butyric acid (IBA - 1.5 mg/L).

Fari *et al*. (1990) studied that the factors like explants and media components, genotypes also play an important role.

Mathew *et al*. (2010) reported that the shoot regeneration potential was comparable in Mint genotypes. In all genotypes IBA stimulated shoot regeneration however, some moderate regeneration was observed even without IBA. In contrast to this, differential *in vitro* regeneration response was observed between six cultivars and one Guatemalan wild accession. The wild accession exhibited the best regeneration response. Out of six cultivars, occasional elongation of shoots in 'Yolo wonder L.' was achieved by incubating cultures in the dark on a medium containing 3 mg/l BA and 1 mg/l IBA.

Cleark *et al*. (2014) obtained maximum number of shoots on MS medium with 2.0 mg/l BA in Mint after 6 weeks of culture, good rooting was observed on IBA + BA or kinetin media in both the species.

Szasz *et al*. (1995) investigated the differential shoot regeneration ability of pepper Mint genotypes on media containing BAP + IBA in addition to standard inbred lines (No. 40017, R-13). Only regenerated shoots but induced buds formed pale leaf like structures on shoot elongation medium. Different responses to regeneration were observed even within the *Mentha sp* species.

2.5 Root induction in explant

Daud *et al*. (2015) carried out an experiment to establish optimum culture condition and to identify the most responsive explants to regenerate *Mentha spp.* through plant tissue culture system. The explants were induced with manipulation of growth regulators during organogenesis. Plant growth regulators that were used are BA and IBA. Different combinations and concentrations of plant growth regulators were added into the MS medium. The new plantlets were raised in a short period of time when stem and root explants were cultured on MS medium containing 2.0 mg/1 BA and 1.0 mg/1 IBA. The well rooted *in vitro* raised plantlets were successfully transferred to soil and their survival rate under natural environment was 90%.

Sujana *et al.* (2011) stated that the rooted plantlets were hardened in polythene cups containing sterile soil and vermiculite (1:1). Plantlets thus developed were successfully established and finally transferred to a greenhouse. The present regeneration system is highly reproducible and the regenerated plants developed normally and were phenotypically similar to the parent plant.

Rajam *et al*. (1996) reported that in tissue cultures of Mint on the shoot induction medium normally roots are not induced. Therefore, rooting has to be induced by subculturing on medium with auxins.

Kiran et. al. (2004) developed a protocol for rapid regeneration from shoot tips and nodal explants of *Mentha piperita* L. on Musashige and Skoog's medium supplemented with either 6-Benzyl amino purine (1 mg/l) or zeatin (0.25 mg/l). The highest number of shoot (49.8) was obtained on medium containing BAP. The regenerated dwarf shoots were further elongated on MS medium supplemented with gibberellic acid (GA3; 1mg/l). *In vitro* shoot were then excised from shoot climps and transferred to rooting medium containing naphthalene acetic acid (1mg/l).

Mark *et al*. (2011) proposed that the supplementation of the shooting medium with 0.05 mg/l IBA and 0.1 mg/l NAA for regeneration of Mint.

Ediba *et al*. (2001) examined that the combined effect of 0.5 mg/l lAA and 0.4 mg/l NAA on root induction.

Zhou *et al*. (1994) obtained that the rooting on half MS with 0.1 mg/l NAA, 0.2 mg/l IAA and 0.5 mg/l IAA for the regeneration of Mint.

Kim *et al*. (2001) observed that the optimal rooting condition for Mint in MS with 0.3 mg/l NAA.

2.6 Hardening and establishment of the regenerated plant

The rooted plantlets were hardened on MS basal liquid medium and subsequently in poly cups containing sterile soil and vermiculite (1:1). Plantlets, thus, developed were successfully established and finally transferred to a green house. The plantlets showed high survival rate (90%) in the soil.

Subhash *et al*. (2015) studied that the regenerated plantlets could be maintained on MS basal medium for 2 weeks and then transferred to vermiculite irrigated with mineral nutrient solution to establish regenerated plant. Szasz *et al*. (1995) reported that *in vitro* rooted plants were acclimatized in a Terrulite vegetable plug mix soil : vermiculite (50:50 v /v), perlite : soil (1:1) mix and then transferred to soil.

Farahani (2013) stated that for proper hardening, plantlets were placed in pots containing 82 cm^3 of a mixture pit moss + perlite (2:1) as acidic soil. During hardening of *in vitro* plantlets, they were transferred to the greenhouse environment (photon flux density of 458 μ molm–2s–1 and humidity 70% to 80%).

CHAPTER III

MATERIALS AND METHOD

3.1 Time and location of the experiment:

The present research 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 June, 2017 to January, 2018.

3.2 Experimental materials:

3.2.1 Source of materials:

The planting materials of *Mentha spicata* L (Mint) were collected from Horticultural Farm, SAU, Agargaon Nursery, Sher-e-Bangla Nagar, Dhaka-1207.

3.2.2 Plant materials:

The nodal segments of *Mentha spicata* L (Mint) were used as experimental materials in the present research work.Then, shoot with young leaves were collected from the Mint plants. The extra leaves were removed and shoot were trimmed to size of 1-2 cm for further use as explant. The explants were washed thoroughly with running tap water for removing soil from root**.**

3.2.3 Instruments:

Metal instruments viz., forceps, scalpals, needles, spatulas and 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. All instruments were sterilized in an autoclave at a temperature of 121 ^{0}C for 20 minutes at 1.06 kg/cm^{2} (15 PSI) pressure.

3.2.4 Glassware:

In all the experiments the borosil glassware was used given priority. The glassware were first rinsed with the liquid cleaners and then washed thoroughly with tap water before the detergent (trix) was removed completely. Then set up to autoclave for sterilization.

3.2.5. 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 has been shown in. Hormones were added separately to different media according to the requirements.

3.2.6 Sterilization of Instruments and Glassware

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

3.3 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.

3.3.1 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.

3.3.2 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.

3.3.3 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.

3.3.4. 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.

3.3.5. Hormone stock solution

The first step of 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 for *in vitro* regeneration of are presented below:

1. BA (1.0, 1.5, 2.0, 2.5 mg/l) for shoot induction

2. BA (1.0, 1.5, 2.0, 2.5 mg/l) combined with IBA 0.5, 1.0, 1.5 and 2.0) for shoot and root formation respectively

3. IBA (0.5, 1.0, 1.5 and 2.0 mg/l) for root induction

3.4 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.

Hormone (solute)	Solvents used
BA	1N NaoH
IBA	70% ethyl alcohol

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

3.4.1 Stock solution of BA

A 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.

3.4.2 Stock solution of IBA

A 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 ± 10^{0}C for use up to two month.

3.5 Treatments

Five sub experiments were conducted to assess the effect of different concentrations of BA and IBA on shoot proliferation and subsequent rooting of the multiplied shoot and BA with NAA for callus induction.

Sub-experiment 1.

Effect of BA on *in vitro* shoot induction potentiality in Mint

Four levels of BA (1.0, 1.5, 2.0 and 2.5 mg/l) and control (0.0 mg/l) treatments were used. The experiments were arranged in Completely Randomized Block (CRD) with three replications.

Sub-experiment 2.

Combined effect of BA and IBA on *in vitro* shoot and root induction potentiality in Mint

Treatments:

In this sub-experiment, Four levels of IBA (0.5, 1.0, 1.5 and 2.0 mg/l) were practiced with each level of BA (1.0, 1.5, 2.0 and 2.5 mg/l). Total 16 combinations of BA and IBA were examined in this experiment and control treatment also practiced. The combine treatments were as follows:

T1 = BA 1.0 mg/l + 0.5mg/l IBA

T2 = BA 1.0 mg/l + 1.0 mg/l IBA

T3 = BA 1.0 mg/l + 1.5 mg/l IBA

T4 = BA 1.0 mg/l + 2.0 mg/l IBA

T5 = BA 1.5 mg/l + 0.5 mg/l IBA

T6 = BA 1.5 mg/l + 1.0 mg/l IBA

T7 = BA 1.5 mg/l + 1.5 mg/l IBA

T8 = BA 1.5 mg/l + 2.0 mg/l IBA

T9 = BA 2.0 mg/l + 0.5 mg/l IBA

T10 = BA 2.0 mg/l + 1.0 mg/l IBA

T11 = BA 2.0 mg/l + 1.5 mg/l IBA

T12 = BA 2.0 mg/l + 2.0 mg/l IBA

T13 = BA 2.5 mg/l + 0.5 mg/l IBA

T14 = BA 2.5 mg/l + 1.0 mg/l IBA

T15 = BA 2.5 mg/l + 1.5 mg/l IBA

T16 = BA 2.5 mg/l + 2.0 mg/l IBA

The experiments were arranged in Completely Randomized Design (CRD) with three replications. Each of replications consisted of five culture vials.

Sub-experiment 3.

Effect of IBA on root induction potentiality of *in vitro* regeneration in Mint

Four levels of IBA (0.5, 1.0, 1.5 and 2.0 mg/l) and control (0.0 mg/l) were used. The experiments also practiced as sub-experiment 1.

Sub-experiment 4.

Acclimatization and establishment of plantlets on soil

Tissue culture derived plantlets were acclimatized in shade house and natural condition to find out the survival percentage.

3.6 Preparation of culture media from MS powder

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

I. 700 ml of sterile distilled water was poured into 1000 ml beaker.

II. 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.

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

IV. The volume was made up to 1000 ml with addition of sterile distilled water.

V. The pH was adjusted at 5.8.

VI. 8 gm agar was added to the mixture and heated for 10 minutes in an electric oven for melting of agar.

VII. Required volume of hot medium was dispensed into culture vessels. After dispensing and proper cooling of the medium, the culture vessels were plugged with cork and marked with different codes with the help of a glass marker to indicate specific hormonal combinations.

3.7 Steam heat sterilization of media (Autoclaving)

For sterilization the culture medium was poured in 200 ml culture bottles and then autoclaving was carried out at a temperature of 121^0C for 20 minutes at 1.06 kg/cm2 (15 PSI) pressure. After autoclaving the media were stored in at 23±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 sprayed with formaldehyde and then the room was kept closed for 3 days. After that 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 and sterilization

The explants (nodal segment) were washed thoroughly under running tap water and then autoclaved with 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 minute and rinsed with autoclaved distilled water for 3- 4 times. After treating with 70% ethanol, the explants were surface sterilized with a 0.1% mercuric chloride solution 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.

3.10 Inoculation of explant in culture media

For inoculation, the workers 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 (0.5-1 cm), explants were transferred to culture bottles containing 20 mL MS medium with plant growth regulator. 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. 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 tissue

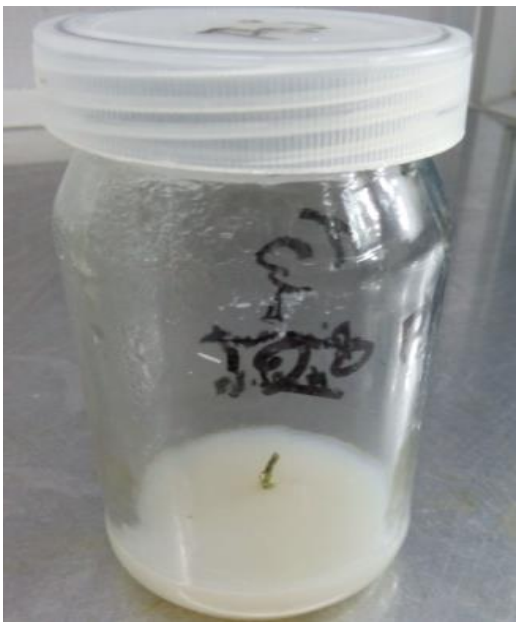

Plate 1: Inoculation of explant in culture media

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 21 ± 1^{o} C by an air conditioner and a 16 hours photo period was maintained along with light intensity of 3000-5000 lux for proper growth and development of culture.

3.12 Sub-culturing and maintaining of proliferating shoots

Initial sub-culturing 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. 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.

3.13 Transfer of plantlets from culture vials to soil

After completing two and half months, the culture vials with well developed plantlets were transferred to normal room temperature. Then next 2-3 days, the rooted plantlets were removed from culture vials and the medium attached to root was gently washed out with tap water. Plantlets were individually transplanted in Plastic pot containing the mixture of soil, sand and cow dung (1:1:1). Immediately after transplantation, the plants along with pot were covered with moist and transparent poly bag for 7 days to prevent desiccation. To reduce sudden shock, the plantlets were kept in shade house for 12 days. Then after 12 days plantlets were transferred to the field.

3.14 Data recording

The observations on development pattern of shoots and roots were made throughout the entire culture period. Three replicates (single shoot per culture bottle) were used per treatment. Data were recorded after 3, 4 and 6 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. No. of shoots per explants
3. Average Length of shoot (cm)

4. Days to root induction

5. No. of roots per explants

6. Average length of root (cm)

7. Percent of explants showing shoot induction

8. Percent of explants showing root induction

3.14.1 Root formation of regenerated shoots

Newly formed shoots with adequate length were excised individually from the culture vial and transferred to rooting media. Growth regulator (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.

3.14.2 Calculation of percentage of shoot induction

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

$$\text{Percentage of shoot induction} = \frac{\text{Number of explant induced shoots}}{\text{Number of explants inoculated}} \times 100$$

3.14.3 Days to shoot induction

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

3.14.4 Number of shoot per explant

Number of shoot per explant was calculated by using the following formula,

$$\text{Number of shoot per explant} = \frac{\text{Number of shoots per explant}}{\text{Number of observation}} \times 100$$

3.14.5 Number of leaf

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

3.14.6 Percent of explants showing root induction

The number of roots were produced per explant were recorded and the percentage of root regeneration was calculated as

$$\text{Percentage of root induction} = \frac{\text{Number of shoot induced root}}{\text{Number of shoot incubated}} \times 100$$

Number of days required for initiation of root from the day of inoculation was recorded.

3.14.7 Number of roots/plantlet

Average number of roots/plantlet was calculated by using formula.

3.14.8 Length of roots

Root length was determined in centimeter (cm) from the base to tip of the roots. Average length of the root was calculated by using formula.

3.14.9 Percentage of established plantlets

The percentages of established plantlets were calculated based on the number of plantlets placed in the plastic pots and the number of plants finally survived. The percentages of established plantlet were calculated by using the following formula:

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

3.14.10 Calculation of number of shoot and root per plant

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

$$\text{Number of shoot per plant} = \frac{\text{Number of shoot / root per explant}}{\text{Number of observation}} \times 100$$

3.14.11 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.

3.14.12 Calculation of survival rate of plantlets:

The survival rate 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-

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

3.15 Statistical data analysis

Data recorded for different parameters under study were statistically analyzed to ascertain the significance of the experimental results. The means for all the treatments were calculated and analyses of variance of all the characters were performed. Experiment was conducted in laboratory and arranged in Completely Randomized Design (CRD) with three replications. The significant difference between the pair of means was evaluated at 1% level of significance by Duncan's Multiple Range Test (DMRT) (Gomez and Gomez, 1984).

CHAPTER IV

RESULT AND DISCUSSION

Different investigations were made on this experiment under laboratory condition to evaluate the effect of different plant growth regulators on multiple shoot and root induction in Mint. Manipulating the relative ratio of different growth regulators has been successfully used in the current investigation. The results obtained from the experiment were described and discussed here and the analyses of varience (ANOVA) are presented in Appendix I-XII. Presentation of results has been made in three phases.

4.1 Sub-experiment 1. Effect of BA on shoot induction potentiality in Mint (*Mentha spicata* L.)

The result of different concentration of BA has been presented under following headings with Figure (1.1-1.2) and Table (1-3).

4.1.1 Days to shoot initiation

Significant variations were observed among different concentrations of BA on days to shoot induction. The maximum 18.00 days to shoot induction were recorded in control treatment followed by the treatment 2.5 mg/l BA (14 days) and 1.0 mg/l BA (13.25 days). On the other hand, minimum 10.25 days was required in the treatment 2.0 mg/l BA followed by 1.5 mg/l of BA (12.75 days) (Figure 1.1).

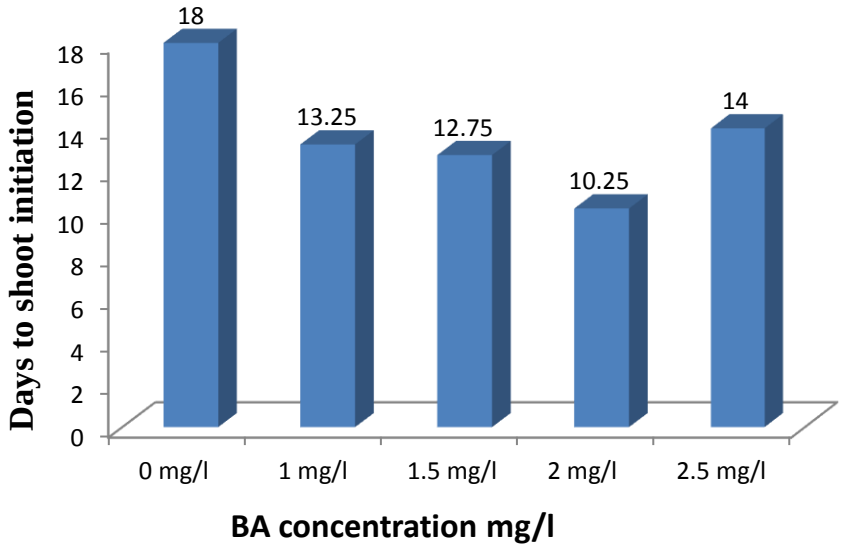

Figure 1.1 Effect of BA on days to shoot induction in Mint

4. 1.2 Percentage of shoot initiation

The different concentrations of BA showed the significant variations on percentage of shoot induction. The treatment BA 2.0 mg/l had produced the highest frequency of shoot (80.00%), while the lowest percentage (40.00%) in control treatment (Figure 1.2).

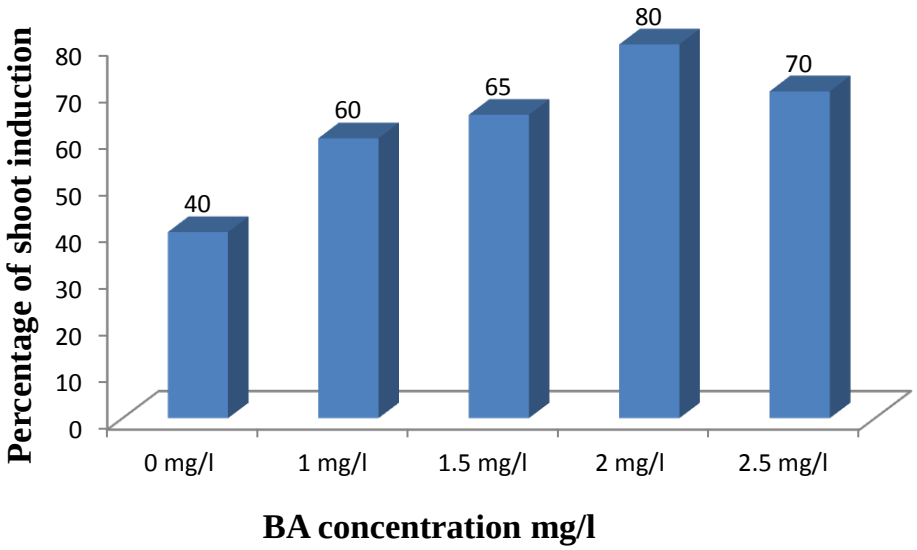

Figure 1.2 Effect of BA on percentage of shoot induction in Mint

4.1.3 Number of shoot per plant

There was a significant influence of different concentrations of BA on the number of shoot at 5% level of variation. The highest number of shoot (8.25, 14.25 and 19.75) at 21, 28 and 42 DAI respectively (Plate 2) was noticed from the treatment 2.0 mg/l BA. Others BA concentration of 2.5 mg/l (5.50, 8.75 and 13.00) and 1.5 mg/l (3.00, 4.50 and 6.50) at 21, 28 and 42 DAI which was statistically similar with each other (Table 1) . Whereas the lowest number of shoot (2.00, 2.50 and 2.75) at 21, 28 and 42 DAI, respectively were noticed in control without hormone (Table 1). Van (2009) found that the highest shoot multiplication successes were obtained in Mint with the treatment of 2.0 ml/l BA for considerable shoot length. Heidari *et al.* (2012) reported that the highest number of shoots per explant in Mint using 2.0 mg/l of BA. The findings of this study are partially similar with their results.

Plate 2. Highest number of shoot induction in MS medium supplemented with BA 2.0 mg/l at 42 DAI

Table 1. Effect of different concentration of BA on number of shoot at different DAI

BA (mg/l)	Number of Shoot/explants		
	21 DAI	28 DAI	42 DAI
0	2.00 d	2.50 d	2.75 d
1.0	3.00 c	4.5 c	6.50 d
1.5	5.25 b	7.75 b	11.50 c
2.0	8.25 a	14.25 a	19.75 a
2.5	5.50 b	8.75 b	13.00 b
CV(%)	12.12	6.05	3.77
LSD(0.05)	1.472	1.178	1.057

Figures in a column followed by different letter(s) differs significantly whereas figures having common letter(s) do not differ significantly from each other as adjusted by DMRT. CV= Coefficient of variation, LSD (0.05) = Least significant difference.

4.1.4 Length of shoot

Significant variation of different concentrations of BA on average length of shoot was found. The highest length of shoot (5.75 cm, 8.75cm and 12.12 cm) at 21, 28 and 42 DAI respectively was noticed from the 2.0 mg/l BA (Plate 3) which was statistically different from rest of treatments. Whereas, the minimum length (2.5cm, 2.75 cm and 2.90 cm) at 21, 28 and 42 DAI respectively were noticed in control treatment (Table 2). Ghanti *et al*. (2004) who reported that the highest length of shoots was observed in Mint by using 2.0 mg/l of BA.

Table 2. Effect of different concentration of BA on length of shoot at DAI

BA (mg/l)	Length of shoot		
	21 DAI	28 DAI	42 DAI
0	2.50 d	2.75 d	2.90 d
1.0	3.00 b	4.25 d	6.625 c
1.5	3.75 b	6.00 c	9.37 b
2.0	5.75 a	8.75 a	12.12 a
2.5	4.50 b	7.62 b	9.25 b
CV (%)	8.25	6.65	6.20
LSD (0.05)	0.823	0.978	1.278

Figures in a column followed by different letter(s) differs significantly whereas figures having common letter(s) do not differ significantly from each other as adjusted by DMRT. CV= Coefficient of variation, LSD(0.05)= Least significant difference.

Plate 3. Length of shoot at 42 DAI in the treatment of 2.0 mg/l BA

4.1.5 Number of leaves

There was a significant influence of different concentrations of BA on the number of leaves per shoot. BA 2.0 mg/l gave the maximum number of leaves (6.00, 8.75and 14.25) (Plate 4) and the second highest leaves number (1.80, 3.20 and 5.20) was found in BA 1.5 mg/l at 21, 28 and 42 DAI respectively (Table 3) whereas, the control treatment showed the lowest number of leaves (1.75, 2.25 and 2.50) at 21, 28 and 42 DAI (Table 3) respectively.

Table 3. Effect of different concentration of BA on number of leaves at Different DAI

BA (mg/l)	Number of leaves		
	21DAI	28DAI	42DAI
0	1.75 d	2.25 d	2.50 c
1.0	3.00 b	4.75 c	7.75 b
1.5	3.75 b	6.00 b	8.00 b
2.0	6.00 a	8.75 a	14.25 a
2.5	3.25 b	5.50 bc	8.75 b
CV (%)	10.21	6.53	7.74
LSD (0.05)	0.901	0.901	1.656

Figures in a column followed by different letter(s) differs significantly whereas figures having common letter(s) do not differ significantly from each other as adjusted by DMRT. CV= Coefficient of variation, LSD (0.05) = Least significant difference.

Plate 4. Number of leaves at 42 DAI in the treatment of 2.00 mg/l BA

4.2 Sub-experiment 2. Effect of IBA on root induction potentiality in Mint

4.2.1 Days to root initiation

Significant variations were observed among different concentrations of IBA on days to root induction. The maximum days (20.5 days) to root induction were recorded in control treatment followed by 2.0 mg/l IBA (16.50 days) and 0.5 mg/l (15.25 days). On the other hand, minimum (12.25 days) was required in 1.0 mg/l IBA followed by 1.5 mg/l (15.50 days) (Figure 2.1). Bariya and Pandya (2014) described that *in vitro* regenerated shoots were shifted to MS medium augmented with 1.0 mg/l indole butaric acid (IBA) for rooting after 4 weeks of sub-culturing. This result may be varied due to the differences of genotype and culture environment (Bolouk *et al*, 2013).

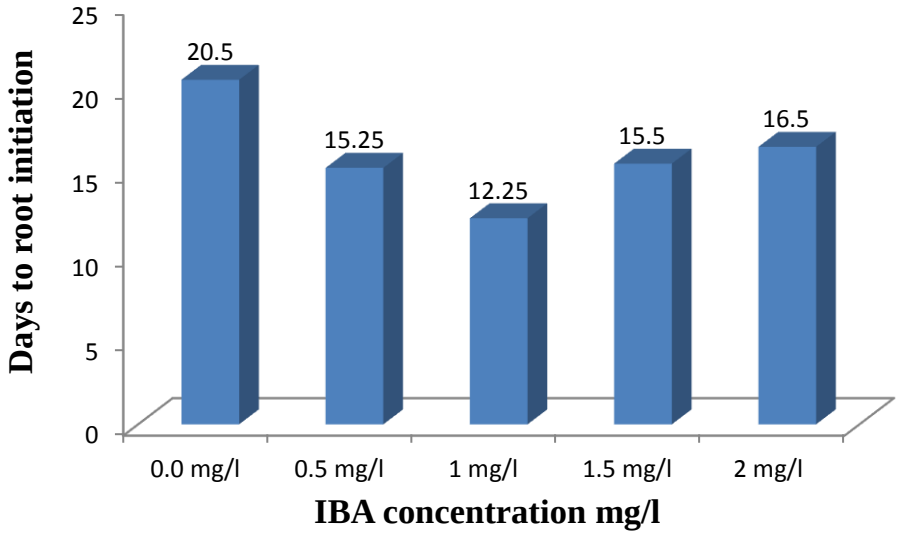

Figure 2.1 Effect of BA on days to root induction in Mint

4.2.2 Percentage of root initiation

The treatment IBA 1.0 mg/l had produced the highest percentage of root induction (78.00%), while the lowest percentage (36.00%) of root induction was produced in control treatment (Figure 2.2). Sharma *et al*. (2012) observed 80% rooting at 1.0 mg/l IBA. Variation may be due to the age, nature, origin

and the physiological state of the explant and seasonal variation play a crucial role in the establishment of cultures and subsequent plant regeneration (Bajaj, Y.P.S. *et al*., 1991)

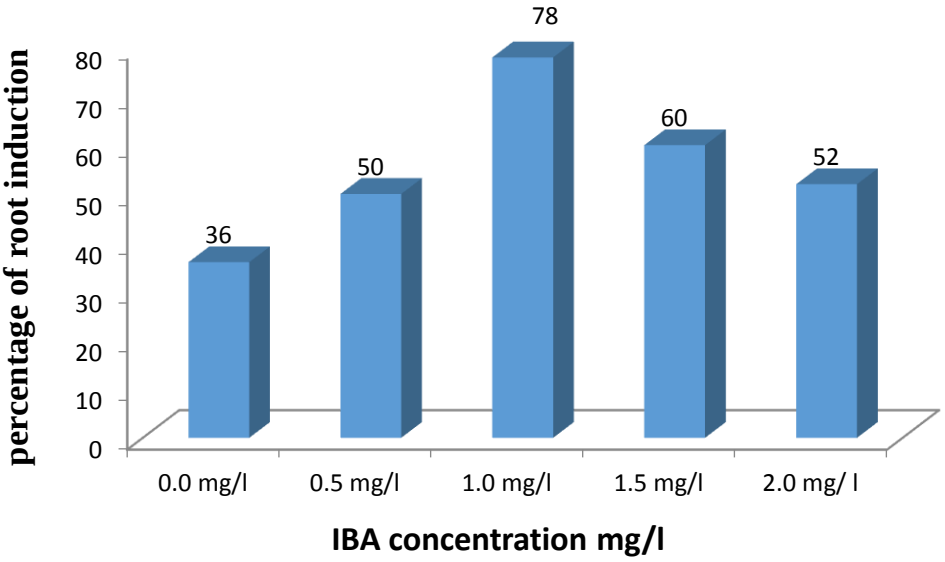

Figure 2.2 Effect of IBA on percentage of root induction in Mint

4.2.3 Number of root per plantlet

There was significant influence of different concentrations of IBA on the number of roots per shoot. IBA 1.0 mg/l gave the highest number of root (7.25, 11.50 and 16.25) at 21, 28 and 42 DAI (Plate 5) and the control treatment was found with the lowest number of root (1.00, 1.20 and 1.40) at 14, 21 and 28 DAI (Table 4). Hoque, (2013) stated that shoots were separated carefully and were transferred to the fresh half strength MS solid medium with indole-3-butyric acid for the development of the roots.

Plate 5. Number of root development in the treatment of IBA 1.00 mg/l

Table 4. Effect of different concentration of IBA on number of root at different DAI

IBA (mg/l)	Number of root per plantlet		
	21DA	28DAI	42DAI
0	1.50 d	1.75 d	2.25 d
0.5	2.50 d	3.50 d	5.75 d
1.0	7.25 a	11.50 a	16.25 a
1.5	4.50 b	7.25 b	11.75 b
2.0	3.75 c	5.5 c	8.25 c
CV (%)	7.41	9.68	8.69
LSD (0.05)	0.736	1.484	2.016

Figures in a column followed by different letter(s) differs significantly whereas figures having common letter(s) do not differ significantly from each other as adjusted by DMRT. CV= Coefficient of variation, LSD(0.05)= Least significant difference.

4.2.4 Length of root

There was a influence of different concentrations of IBA on the length of root at 5% level of significance. The highest length of root (7.50 cm) at 42 DAI (Plate 6) was noticed from the 1.0 mg/l IBA followed by 0.5 mg/l (4.37 cm)

and 2.0 mg/l (3.62cm). On the other hand, the lowest length of root (1.00 cm) at 42 DAI was noticed in control without hormone followed by 2.0 mg/l (3.62 cm) (Table 05).

Table 05. Effect of different concentration of IBA on length of root at different DAI

IBA (mg/l)	Length of root		
	21DAI	28DAI	42DAI
0	1.00 c	1.25 c	1.50 c
0.5	2.00 b	3.25 a	4.37 b
1.0	3.25 a	5.00 a	7.50 a
1.5	2.25 b	3.20 b	4.62 b
2.0	1.75 b	2.50 b	3.62 b
CV (%)	11.95	13.04	10.77
LSD (0.05)	0.610	1.008	1.196

Figures in a column followed by different letter(s) differs significantly whereas figures having common letter(s) do not differ significantly from each other as adjusted by DMRT. CV= Coefficient of variation, LSD (0.05)= Least significant difference.

Plate 6. Length of root at 42 DAI in the treatment of 1.00 mg/l IBA

4.3 Sub-experiment 3. Effect of BA and IBA on shoot and root induction potentiality in Mint

The results of the combined effect of different concentrations of BA + IBA have been presented under following headings with Table (6-12) and Plate (8-12).

4.3.1 Days to shoot initiation

Significant variations were observed for the different concentrations of BA + IBA on days to shoot induction. The minimum duration 8.33 days was obtained in BA 2.0 mg/l+ IBA 1.0 mg/l than rest of the treatments. On the other hand, the maximum days (16.50 days) to shoot induction was recorded in control (Table 6).Which is partially similar with the findings of Bolouk *et at* (2013) for Mint shoot induction with BA 2.0 mg/l + IBA 0.5 mg/l (8.66 days) (Table 6).

4.3.2 Percentage of shoot initiation

There was a significant influence of different concentrations of BA + IBA on the percentage of shoot induction per explant. The optimum percentage (85.00%) of shoot induction was noticed in treatment BA 2.0 mg/l + IBA 1.0 mg/l which was best than other treatment and minimum percentage (35.00%) was noticed in control treatment hormone free media (Table 6). Kane, M. (2014) observed that the treatment 2.0 mg/l BA and 1.0 mg/l IBA produced optimum percentage of shoot organogenesis in Mint after 6 weeks of sub-culturing. This variation may be due to growth regulators in the culture media, genetic, physiological and morphological change in *in vitro* (Chaturvedi *et al*. 2007).

Table 06. Effect of different concentration of BA and IBA on days to shoot initiation and percent of shoot initiation

Treatment	Days to shoot initiation	Percent of shoot initiation (%)
Control	16.50	35.00
BA 1.0mg/l+ IBA 0.5 mg/l	12.66	55.00
BA 1.0mg/l+ IBA 1.0 mg/l	9.66	81.00
BA 1.0mg/l+ IBA 1.5 mg/l	12.33	66.6
BA 1.0mg/l+ IBA 2.0 mg/l	13.66	61.66
BA 1.5mg/l+ IBA 0.5 mg/l	12.00	60.00
BA 1.5mg/l+ IBA 1.0 mg/l	8.66	83.00
BA 1.5mg/l+ IBA 1.5 mg/l	12.33	70.00
BA 1.5mg/l+ IBA 2.0 mg/l	13.66	63.33
BA 2.0mg/l+ IBA 0.5 mg/l	11.33	60.00
BA 2.0mg/l+ IBA 1.0 mg/l	8.33	85.00
BA 2.0mg/l+ IBA 1.5 mg/l	12.33	70.00
BA 2.0mg/l+ IBA 2.0 mg/l	13.33	61.66
BA 2.5mg/l+ IBA 0.5 mg/l	13.00	56.66
BA 2.5mg/l+ IBA 1.0 mg/l	9.00	78.33
BA 2.5mg/l+ IBA 1.5 mg/l	12.66	68.33
BA 2.5mg/l+ IBA 2.0 mg/l	14.00	61.66
CV (%)	4.67	3.55
LSD(0.05)	1.67	7.30

Figures in a column followed by no letter(s) differs significantly whereas figures having common letter(s) do not differ significantly from each other as adjusted by DMRT. CV= Coefficient of variation, LSD(0.05)= Least significant difference.

4.3.3 Number of shoot per plant

Different concentrations of BA and IBA showed significant variations on the number of shoot. The highest number of shoot (10.00, 14.67 and 20.33) was noticed from the BA 2.0 mg/l + IBA 1.0 mg/l (Plate 7) and second highest number (9.67, 14.33 and 19.67) at 21, 28 and 42 DAI respectively, were observed in 2.5 mg/l BA + 1.0 mg/l IBA. Whereas the lowest average number of shoot (3.00, 3.75 and 4.5) at 21, 28 and 42 DAI respectively were noticed in control treatment (Table 7). These findings are partially similar to the results of Sharma *et al*. (2012). They found that each inoculated explant produced 18.10 ± 0.66 shoots within 2 to 3 weeks from *in vitro* grown plantlets inoculated on the Murashige and Skoog (MS) medium supplemented with 4.44 μM BA in combination with 2.85 μM IBA in Mint.

Table 7. Effect of BA and IBA on number of shoot at different DAI

Treatment	Number of shoot/explants		
	21DAI	**28DAI**	**42DAI**
Control	3.00 g	3.70 g	4.50 g
BA 1.0mg/l+ IBA 0.5 mg/l	4.00 e	5.66 e	8.00 f
BA 1.0mg/l+ IBA 1.0 mg/l	9.66 a	14.66 a	19.66 a
BA 1.0mg/l+ IBA 1.5 mg/l	6.33 bc	9.00 bc	13.33 b-d
BA 1.0mg/l+ IBA 2.0 mg/l	5.33 c-e	7.66 cd	11.33 e
BA 1.5mg/l+ IBA 0.5 mg/l	4.66 de	6.66 de	9.00 f
BA 1.5mg/l+ IBA 1.0 mg/l	9.66 a	13.67 a	19.67 a
BA 1.5mg/l+ IBA 1.5 mg/l	7.00 b	10.33 b	14.66 b
BA 1.5mg/l+ IBA 2.0 mg/l	6.00 b-d	9.00 bc	12.66 c-e
BA 2.0mg/l+ IBA 0.5 mg/l	4.00 e	6.00 e	9.00 f
BA 2.0mg/l+ IBA 1.0 mg/l	10.00 a	14.67 a	20.33 a
BA 2.0mg/l+ IBA 1.5 mg/l	7.00 b	10.00 b	14.33 bc
BA 2.0mg/l+ IBA 2.0 mg/l	6.00 b-d	8.67b-d	13.00 b-e
BA 2.5mg/l+ IBA 0.5 mg/l	4.00 e	6.00 e	8.00 f
BA 2.5mg/l+ IBA 1.0 mg/l	9.66 a	14.33 a	19.66 a
BA 2.5mg/l+ IBA 1.5 mg/l	6.66 bc	9.33 bc	13.33 b-d
BA 2.5mg/l+ IBA 2.0 mg/l	5.66 b-d	8.33 b-d	11.67de
CV (%)	6.91	7.65	4.49
LSD(0.05)	1.393	2.252	1.866

Figures in a column followed by different letter(s) differs significantly whereas figures having common letter(s) do not differ significantly from each other as adjusted by DMRT. CV= Coefficient of variation, LSD(0.05)= Least significant difference.

Plate 7. Number of shoot at 42 DAI in the treatment of BA 2.00 mg/l + IBA 1.00 mg/l

4.3.4 Length of shoot (cm)

There was a variation in different concentrations of BA with IBA on the length of shoot at 5% level of significance. The highest length of shoot (6.16 cm, 8.30 cm and 13.00 cm) at 21, 28 and 42 DAI, respectively was noticed from the BA 2.0 mg/l + IBA 1.0 mg/l (Plate 8) followed by BA 1.5 mg/l + IBA 1.0 mg/l (6.16 cm, 8.33 cm and 12.83 cm) and BA 1.0 mg/l+ IBA 1.0 mg/l (6.00, 8.12 and 12.5 cm) at 21, 28 and 42 DAI respectively. Whereas the minimum length of shoot (1.75 cm, 2.00 cm and 2.25 cm) at 21, 28 and 42 DAI respectively were noticed in control treatment (Table 8).

Table 8. Effect of different concentration BA and IBA on length of shoot

Treatments	length of shoot (cm)		
	21DAI	**28DAI**	**42DAI**
Control	1.75 f	2.00 f	2.25 f
BA 1.0mg/l+ IBA 0.5 mg/l	3.33 de	4.83 ef	6.83 e
BA 1.0mg/l+ IBA 1.0 mg/l	6.16 a	8.12 a	12.63 a
BA 1.0mg/l+ IBA 1.5 mg/l	5.00 b	7.00 bc	9.8 b
BA 1.0mg/l+ IBA 2.0 mg/l	4.66 bc	6.33 cd	9.0 c
BA 1.5mg/l+ IBA 0.5 mg/l	3.00 e	4.33 f	6.50 ef
BA 1.5mg/l+ IBA 1.0 mg/l	6.33 a	8.33 a	12.83 a
BA 1.5mg/l+ IBA 1.5 mg/l	5.00 b	7.00 bc	9.83 b
BA 1.5mg/l+ IBA 2.0 mg/l	4.33 bc	6.33 cd	9.0 c
BA 2.0mg/l+ IBA 0.5 mg/l	4.00 cd	5.50 de	8.00 d
BA 2.0mg/l+ IBA 1.0 mg/l	6.16 a	8.33 a	13.00 a
BA 2.0mg/l+ IBA 1.5 mg/l	4.66 bc	6.33 cd	9.16 bc
BA 2.0mg/l+ IBA 2.0 mg/l	3.83 c-e	5.00 ef	8.00 d
BA 2.5mg/l+ IBA 0.5 mg/l	3.00 e	4.16 f	6.00 f
BA 2.5mg/l+ IBA 1.0 mg/l	6.00 a	8.00 ab	12.50 a
BA 2.5mg/l+ IBA 1.5 mg/l	4.33 bc	6.33 cd	9.33 bc
BA 2.5mg/l+ IBA 2.0 mg/l	4.00 cd	5.50 de	8.00 d
CV (%)	6.84	5.95	6.43
LSD(0.05)	0.960	1.149	0.696

Figures in a column followed by different letter(s) differs significantly whereas figures having common letter(s) do not differ significantly from each other as adjusted by DMRT. CV= Coefficient of variation, LSD(0.05)= Least significant difference.

Plate 8. Length of shoot At 42 DAI in the treatment of BA 2.00 mg/l + IBA 1.00 mg/l

4.3.5 Number of leaves

The number of leaves per shoot showed significant difference with combined concentrations of BA and IBA. The treatment BA 1.5 mg/l+ IBA 1.0 mg/l gave the highest number of leaves (9.33, 14.66 and 20.67) at 21, 28 and 42 DAI respectively (Plate 9) whereas the lowest number of leaves (4.00, 4.50 and 5.00) at 21, 28 and 42 DAI respectively was found with hormone free media (Table 9).

Plate 9. Number of leaves at 42 DAI in the treatment of BA 2.00 mg/l + IBA 1.00 mg/l

Table 9. Effect of different concentration BA and IBA on number of leaves

Treatments	Number of leaves		
	21DAI	28DAI	42DAI
Control	4.00 d	4.50 d	5.00 d
BA 1.0mg/l+ IBA 0.5 mg/l	6.00 c	8.67 b-d	13.00 b-e
BA 1.0mg/l+ IBA 1.0 mg/l	8.67 ab	13.33 a	19.67 a
BA 1.0mg/l+ IBA 1.5 mg/l	6.67 c	9.33 b-d	14.00 b-d
BA 1.0mg/l+ IBA 2.0 mg/l	6.33 c	8.67 b-d	13.00 b-e
BA 1.5mg/l+ IBA 0.5 mg/l	6.67 c	9.67 b-d	13.67 b-d
BA 1.5mg/l+ IBA 1.0 mg/l	9.3 a	14.33 a	20.67 a
BA 1.5mg/l+ IBA 1.5 mg/l	7.00 bc	10.67 b	14.67 bc
BA 1.5mg/l+ IBA 2.0 mg/l	5.67 c	8.67 b-d	12.00 de
BA 2.0mg/l+ IBA 0.5 mg/l	5.67 c	8.33 b-d	12.33 c-e
BA 2.0mg/l+ IBA 1.0 mg/l	7.80 b	10.67 b	17.00 b
BA 2.0mg/l+ IBA 1.5 mg/l	7.00 bc	10.33 bc	15.00 b
BA 2.0mg/l+ IBA 2.0 mg/l	5.33 c	8.00 cd	13.00 b-e
BA 2.5mg/l+ IBA 0.5 mg/l	5.33 c	7.67 d	11.00 e
BA 2.5mg/l+ IBA 1.0 mg/l	9.00 a	13.33 a	18.67 a
BA 2.5mg/l+ IBA 1.5 mg/l	6.33 c	9.33 b-d	13.67 b-d
BA 2.5mg/l+ IBA 2.0 mg/l	5.33 c	8.00 cd	11.67 de
CV (%)	9.2	8.43	5.65
LSD(0.05)	1.886	2.548	2.547

Figures in a column followed by different letter(s) differs significantly whereas figures having common letter(s) do not differ significantly from each other as adjusted by DMRT. CV= Coefficient of variation, LSD(0.05)= Least significant difference.

4.3.6 Days to root induction

Significant variation was observed among different concentrations of BA and IBA on days to root induction. The maximum (18.00 days) to root induction was recorded in control treatment and minimum (11.00 days) was required in BA 2.0 mg/l + IBA 1.0 mg/l concentration (Table 10).

4.3.7 Percentage of root induction

Different concentrations of BA and IBA showed the significant variations on percent of explants showing root induction. The highest percentage (85.00%) of root induction was recorded with BA 2.0 mg/l + IBA 1.0 mg/l, whereas the lowest percentage (36.00%) of root induction was recorded in control condition (Table 10).

Table 10. Effect of different concentration BA and IBA on Days to root induction and Percentage of root initiation

Treatment	Days to root induction	Percent of root initiation
control	18.00 a	36.00 g
BA 1.0mg/l+ IBA 0.5 mg/l	13.67 b	55.00 f
BA 1.0mg/l+ IBA 1.0 mg/l	12.00 c	81.00 a
BA 1.0mg/l+ IBA 1.5 mg/l	13.33 b	66.66 b-d
BA 1.0mg/l+ IBA 2.0 mg/l	14.67 b	61.66 c-f
BA 1.5mg/l+ IBA 0.5 mg/l	14.00 b	60.00 d-f
BA 1.5mg/l+ IBA 1.0 mg/l	12.00 c	83.00 a
BA 1.5mg/l+ IBA 1.5 mg/l	14.00 b	70.00 b
BA 1.5mg/l+ IBA 2.0 mg/l	14.33 b	63.33 b-e
BA 2.0mg/l+ IBA 0.5 mg/l	14.33 b	60.00 d-f
BA 2.0mg/l+ IBA 1.0 mg/l	11.00 d	85.00 a
BA 2.0mg/l+ IBA 1.5 mg/l	14.00 b	70.00 b
BA 2.0mg/l+ IBA 2.0 mg/l	14.00 b	61.66 c-f
BA 2.5mg/l+ IBA 0.5 mg/l	14.00 b	56.66 ef
BA 2.5mg/l+ IBA 1.0 mg/l	11.33 d	78.33 a
BA 2.5mg/l+ IBA 1.5 mg/l	13.66 b	68.33 bc
BA 2.5mg/l+ IBA 2.0 mg/l	14.66 a	61.66 c-f
CV (%)	5.39	3.55
LSD(0.05)	2.183	7.307

Figures in a column followed by different letter(s) differs significantly whereas figures having common letter(s) do not differ significantly from each other as adjusted by DMRT. CV= Coefficient of variation, LSD(0.05)= Least significant difference.

4.3.8 Number of root per plant

There was a significant influence of different concentrations of BA and IBA on the number of root per shoot. The treatment BA 2.0 mg/l+ IBA 1.0 mg/l gave the highest number of root (10.00, 15.00 and 20.67) (Plate 10) and second best result (9.67, 14 and 20.67) was noticed from BA 2.0 mg/l + IBA 1.0 mg/l at 21, 28 and 42 DAI whereas the lowest number of root (3.00, 3.50 and 3.75) at 21, 28 and 42 DAI was found with hormone free media (Table 11).

Plate 10. Number of root at 42 DAI in the treatment of BA 2.00 mg/l + IBA 1.00 mg/l

Table 11. Effect of different concentration BA and IBA on number of roots at different DAI

Treatments	Number of roots /explants		
	21DAI	**28DAI**	**42DAI**
Control	3.25 f	3.50 g	3.75 f
BA 1.0mg/l+ IBA 0.5 mg/l	4.67 d	6.67 f	9.67 e
BA 1.0mg/l+ IBA 1.0 mg/l	9.33 a	13.33 b	19.33 a
BA 1.0mg/l+ IBA 1.5 mg/l	6.67 b	9.00 cd	14.00 b
BA 1.0mg/l+ IBA 2.0 mg/l	5.67 b-d	7.67 d-f	11.33 de
BA 1.5mg/l+ IBA 0.5 mg/l	5.00 cd	7.00 f	9.67 e
BA 1.5mg/l+ IBA 1.0 mg/l	9.67 a	14.00 ab	20.00 a
BA 1.5mg/l+ IBA 1.5 mg/l	6.67 b	9.33 c	13.67 b
BA 1.5mg/l+ IBA 2.0 mg/l	5.33 b-d	7.33 ef	11.67 cd
BA 2.0mg/l+ IBA 0.5 mg/l	5.33 b-d	7.33 ef	10.33 de
BA 2.0mg/l+ IBA 1.0 mg/l	10.00 a	15.00 a	20.67 a
BA 2.0mg/l+ IBA 1.5 mg/l	6.33 bc	9.00 cd	13.67 b
BA 2.0mg/l+ IBA 2.0 mg/l	5.33 b-d	7.33 ef	11.67 cd
BA 2.5mg/l+ IBA 0.5 mg/l	4.67 d	6.67 f	9.67 e
BA 2.5mg/l+ IBA 1.0 mg/l	9.67 a	13.67 ab	19.67 a
BA 2.5mg/l+ IBA 1.5 mg/l	6.67 b	8.67 c-e	13.33 bc
BA 2.5mg/l+ IBA 2.0 mg/l	5.00 cd	7.00 f	10.67 de
CV (%)	7.35	9.325	4.56
LSD(0.05)	1.476	1.487	1.902

Figures in a column followed by different letter(s) differs significantly whereas figures having common letter(s) do not differ significantly from each other as adjusted by DMRT. CV= Coefficient of variation, LSD(0.05)= Least significant difference.

4.3.9 Length of root (cm)

There was significant influence of different combined concentrations of BA and IBA on the length of root. There was a significant variation at 21, 28 and 42 DAI among different concentration of BA and IBA. The highest length of root (4.00, 5.33 and 7.67) at 21, 28 and 42 DAI respectively was found in BA 2.0 mg/l+ IBA 1.0 mg/l (Plate 11) which is statistically similar with BA 1.5 mg/l+ IBA 1.0 mg/l (3.88 cm, 5.10 cm and 7.00 cm) at 21, 28and 42 DAI, respectively (Table 10). The control treatment found the lowest number of root (0.75 cm, 1.00 cm and 1.25 cm) at 21, 28 and 42 DAI (Table 12).

Plate 11. Length of root at 42DAI in the treatment of BA 2.00 mg/l + IBA 1.00 mg/l

Table 12. Effect of BA and IBA on length of root at different DAI

Treatments	Length of root		
	21DAI	**28DAI**	**42DAI**
Control	0.75 f	1.00 h	1.25 g
BA 1.0mg/l+ IBA 0.5 mg/l	1.83 ef	2.50 gh	3.50 fg
BA 1.0mg/l+ IBA 1.0 mg/l	3.80 ab	5.13 a	7.46 a
BA 1.0mg/l+ IBA 1.5 mg/l	3.17 bc	4.17 bc	6.16 c
BA 1.0mg/l+ IBA 2.0 mg/l	2.50 c-e	3.50 c-f	5.67 c-e
BA 1.5mg/l+ IBA 0.5 mg/l	2.00 ef	3.00 e-g	4.00 e-g
BA 1.5mg/l+ IBA 1.0 mg/l	3.88 a	5.10 a	7.00 a
BA 1.5mg/l+ IBA 1.5 mg/l	3.17 bc	4.13 bc	6.33 bc
BA 1.5mg/l+ IBA 2.0 mg/l	2.33 de	3.17 d-g	4.67 d-f
BA 2.0mg/l+ IBA 0.5 mg/l	1.50 f	2.00 h	3.00 g
BA 2.0mg/l+ IBA 1.0 mg/l	4.00 ab	5.33 a	7.67 a
BA 2.0mg/l+ IBA 1.5 mg/l	3.00 cd	4.33 b	6.17 c
BA 2.0mg/l+ IBA 2.0 mg/l	2.50 c-e	3.67 b-e	5.33 cd
BA 2.5mg/l+ IBA 0.5 mg/l	1.83 ef	2.83 fg	4.00 e-g
BA 2.5mg/l+ IBA 1.0 mg/l	3.83 ab	5.50 a	7.50 ab
BA 2.5mg/l+ IBA 1.5 mg/l	2.83 cd	3.83 b-d	5.67 cd
BA 2.5mg/l+ IBA 2.0 mg/l	2.00 ef	3.00 e-g	4.00 e-g
CV (%)	8.64	7.13	7.88
LSD(0.05)	0.725	0.831	1.332

Figures in a column followed by different letter(s) differs significantly whereas figures having common letter(s) do not differ significantly from each other as adjusted by DMRT. CV= Coefficient of variation, LSD(0.05)= Least significant difference.

Sub-experiment 4. Acclimatization and establishment of plantlets on soil

After a considerable shoots and roots were developed at 6 -8 weeks of culture, Then the plantlets were removed from vial carefully without any root damage. The roots were washed with running tap water for removing media from plantlets. Then, the small plantlets were taken to growth cabinet for acclimatization and maintained for further observations under controlled conditions providing with light, temperature and reltive humidity to make a favorable condition for plant establishment. In the meantime, the plantlets transferred to vermiculite pot filled with sterilized soil : cowdung (1:1) and soil mixture provided 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). After all, in the growth cabinet and in the shade house, plants are acclimatized and hardened before being transferred to the field conditions. At first 30 plants were transplanted and 25 were survived in shade condition (83.33%). Finally in normal atmospheric condition 25 plants were transplanted among them 20 survived and survival rate was 80%. Sharma *et al*. (2012) found that plantlets of Mint were successfully transferred to the soil where they grew well for 8 to 10 weeks with 80% survivability. So considering the survival rate it can be said that acclimatization potentiality of Mint is satisfactory.

Table 13. Survival rate of *in vitro* regenerated plantlets of Mint

Acclimatization	No. of plants transplanted	No. of plants survived	Percentage of survival rate (%)
In shade area with controlled atmosphere	30	25	83.33
In natural condition	25	20	80

a. Pot in the shade condition

b. In the natural condition

Plate 12. Acclimatization and establishment of plantlets (a) Pot in the shade condition (b) In the natural condition

CHAPTER V

SUMMARY AND CONCLUSIONS

The present research 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 February 2017 to June 2018. The nodes of Mint were used as experimental materials in the present investigation. BA (1.0, 1.5, 2.0 and 2.5 mg/l) and IBA (0.5, 1.0, 1.5 and 2.0 mg/l) alone or combination were used as treatment. The experiments were arranged in Completely Randomized Design (CRD) with three replications

BA 2.0 mg/l gave the highest number of shoot (8.25, 14.25 and 19.75) at 21, 28 and 42 DAI and the control treatment found the lowest number of shoot (2.00, 2.50 and 2.75) at 21 , 28 and 42 DAI. The highest number of leaves (6.00, 8.75 and 14.25) at 21, 28 and 42 DAI respectively was noticed from the 2.0 mg/l BA.

In combine effect, maximum percentage (83.00%) of shoot induction was noticed in treatment BA 2.0 mg/l + IBA 1.0 mg/l and minimum percentage (35.00%) was in control hormone free media. The treatment BA 2.0 mg/l + IBA 1.0 mg/l gave the highest number of shoot (10.00, 14.67 and 20.33) at 21, 28 and 42 DAI respectively. The maximum number of leaves (9.00 cm, 13.66 cm and 20.66 cm) at 21, 28 and 42 DAI respectively was noticed from the combine dose of BA 2.0 mg/l + IBA 1.0 mg/l and the minimum leaves number and length were noticed in control treatment.

The treatment IBA 1.0 mg/l had produced the highest percentage of root induction (78.00%) required minimum (12.25 days). IBA 1.0 mg/l gave the highest number of root (7.25, 11.50 and 16.25) at 21, 28 and 42 DAI and the control treatment found the lowest number of root at all DAI. The highest length of root (3.25, 5.00 and 7.50) at 21, 28 and 42 DAI respectively was found in 1.0 mg/l IBA and the control treatment found the lowest length of root at all DAI.

A convenient protocol of Mint is established. The moderate dose of BA 2.0 mg/l gave the best results for shoot regeneration. In the combined dose of BA 2.0 mg/l with IBA 1.0 mg/l gave the best result. Besides, IBA 1.0 mg/l gave the best performance in case of root regeration. A protocol of *in vitro* rapid regeneration of Mint has been established which may contribute in and large scale virus free seedlings production throughout the year is possible.

RECOMMENDATIONS

Based on the summary and conclusion following recommendations can be made:

i. More explants such as shoot tip, meristem, leaf and root tip can be experimented for *in vitro* regeneration of Mint.

ii. Accurate and details investigation on influence of other factors such as different elicitors, antioxidants should be considered.

iii. Further study can be done with different concentrations and combinations of auxins and cytokinins group of hormones for rapid proliferation of Mint.

REFERENCES

Agarwal, S., Chandra, N. and Lothari, S. L. (2010). Shoot tip culture of *Mentha spicata* for micropropagation. *Cur. Sci.* **57(1)**: 1347-1349.

Agar, S., Micheal., N. and Jim, S. L. (2001). *In vitro* culture of Mint for micropropagation. *Cur. Sci*. **7(1):**117-25.

Ali, G., Fazal, H., Zahir, A., Muhammad, T., Muhamad, A. K. (2007*). In vitro* complete plant propagation of different cultivators of Mint. On media different hormonal concentration, Chakdara, Pakistan. *Crop. Sci*.**11(2):**221-265.

Ali, M. A., M. Saleem, W. Ahmad, M. Parvez and R. Yamdagni.(2002). A chlorinated monoterpene ketone, acylated-sitosterol glycosides and a flavanone. Journal of Phytological Research. **4(1)**:73-77.

Anilkumer. (2010). Shoot tip culture of pepper for micropropagation. *Cur. Sci.* **5(1):** 1347-1349.

Anonymous. (2008). Final Report on Cultivation of mint as a viable alternative livelihood in East and North East of Afghanistan (RALF 02-011). International Center for Agricultural Research in the Dry Areas; Ministry of Agriculture, Irrigation & Livestock and Faculty of Agriculture, Kabul University. pp. 1-44.

Arous, S., M. Boussaïd and M. Marrakchi. (2001). Preliminary research about *in vitro* regeneration Mint. Avignon-France, September. **7(11)**: 187-189.

Arroya, R. and Revilla, M. A. (1991). *In vitro* plant regeneration from cotyledon and hypocotyls segments in two mint cultivars. Plant Cell Reports. **10**: 414- 416.

Auer, S., Chandra, N., and Kothari, S. L. (2002). Plant regenerating in tissue cultures of mint (*Mentha sp*). Plant Cell, Tissue and Organ Culture. **16**: 47-55.

Bajaj, Y.P.S., Springer, V., Berlin and TIiedelberg. (1991). The effect of the nature and origin of explants on micropropagation. *Int. Biot. in Agri. and Forestry*. **17**: 142-167.

BARI (Bangladesh agricultural research institute). (2016). BARI Annul Report 2015-2016.pp. 145.

Bariya, R. R. and H. A. Pandya. (2014). Evaluation and establishment of promising largescale *in vitro* production of corn mint. Intl. J. Recent Scientific Res. **5(2)**:509-512.

Bhat, S., P. Maheshwari, S. Kumar and A. Kumar. (2002). Mentha species: *In vitro* Regeneration and Genetic Transformation. *Molecular Biol. Today*. **3(1)**:11-23.

Bhoite, H.A. and G.S. Palshikar, (2014). Plant tissue culture: A review. *World J. Pharm. Sci.**8(2)***:565-572.

Bolouk, S. G., S. K. Kazemitabar and J. M. Sinaki. (2013). *In vitro* culture of the peppermint plant (Mentha piperita) without the use of hormones. *Intl. J. Agril. Crop Sci.* **6(18)**:1279-1283.

Borkowska B. (2001). Morphological and physiological Characteristics of Micropropagated Strawberry Plant Rooted *in vitro* or ex vitro. Scientia Horticulturae. **89**:195-206.

Chaturvedi, H.C., M. Jain and N.R. Kidwai.(2007). Cloning of medicinal plants through tissue culture. *A review. Ind. J. Experimental Biol.* **45**: 937-948.

Christopher, Fari, M.,and Andrasflaver, A. (2016). Regeneration and cloning of pepper mint (*Mentha spp.). A Review of Hort. Sci.* **26**: 9-27.

Christopher, T. and Rajam, M. V.(2010). *In vitro* clonal propagation of *Mentha spp*. Plant cell, Tissue and Organ Culture. **38**: 25-29.

Cleark. M., and S.L. Kitto. (2014*). In vitro* regeneration of peppermint and orangemint from leaf disks. *Plant Cell Tissue Org. Cult*. **10(1):**11-19.

Caboni, E., Tonelli, M.G. (2009). Effect of 1, 2-benzisoxazole-3-acetic acid on adventitious shoot regeneration and in vivo rooting in Mint. Plant Cell Report. **18**: 985-988.

Daud, N.F.A., Hasbullah, N.A, Azis, N.A, Rasad, F.M, Amin M.A.M, and Lassim, M.M. (2015). *In vitro* Regeneration of *Mentha spp*. through Stems, Roots, Leaves and Petioles Culture. International Conference on Agricultural, Ecological and Medical Sciences (AEMS-2015). April 7-8, Phuket (Thailand).

Ebida, A. I. A. and Hu, C. Y., (2002).*In vitro* morphogenetic response and plant regeneration from Mint seedling explants. Plant Cell Reports. **13(2):** 107-110.

Farhani, V.J.M. and S.L. Kitto. (2013). Regeneration and establishment of peppermint and orangemint from leaf disks. *Plant Cell Tissue Org. Cult.* **30(1):**41-49.

Fari, M. and Andrasflaver, A., (2003) Regeneration and cloning of pepper (Mentha spp.). *A Review of Hort. Sci.* **26**: 9-27.

Fari, M. and Czako, (1986). Relationship between positions and morphogenetic response of pepper mint hypocotyls explants cultured in *In vitro. Sci. Hort.* **15**: 207213.

Foster. (1996). Production of plantlets of *Mentha spp* in *in vitro*. Journal of Phytology Research. **4(1):** 73-77.

Gayathri, A. C. and Rao, P. S., (2015). *In vitro* plant regeneration from hypocotyls and cotyledon explants of Mint. *Plant Sci*. **11**: 365372.

Mark, S., and Fred, A. (2011). Maintenance and multiplication of mint germplasm. *Intl. J. Farming Allied Sci.* **1(2)**:48-53.

George, E.F., (1993). Plant Propagation by Tissue Culture. Eastern Press, Eversley.

Ghanti, K., C. P. Kaviraj, R. B. Venugopal., F. T. Z. Jabeen and S. Rao. (2004). Rapid regeneration of Mentha piperata L. from shoot tip and nodal explants. *Indian J. Biotech*. **3**: 594-598.

Gomez, K.A. and Gomez, A.A. (1984). Statistical ProceduresforAgricultural Research. 2nd Edn John Willey and Sons, New York, USA, ISBN: 680.

Haque, S.M., Paul, S., & Ghosh, B. (2016). High-frequency *in vitro* flowering, handpollination and fruit setting in ten different cultivars of Mint. An initial step towards *in vitro* hybrid production. Plant Cell, Tissue and Organ Culture. 127. 161–173.

Hasanat, J. L. and Godt. M.J. (2007). Allozyme diversity in plant species. In: Plant Population Genetics, Breeding and Genetic Resources eds. Brown AHD, Clegg MT, Kahler AL, Weir BS. pp. 43-63.

Hassan AKMS and Roy SK (2005). Micropropagation of Gloriosa superba L. through high frequency shoot proliferation. Plant Tissue Cult. **15(1)**: 67-74.

Heidari, F., S. Jahanbakhsh, M. Talebi, C. Ghobadi and M. A. S. Kandi. (2012). Regeneration and *in vitro* cultivation of three Iranian Mentha species using meristem, node and leaf disk cultures. *Intl. J. Farming Allied Sci.* **1(2)**:48-53.

Hoque, M. A. (2013). Maintenance and multiplication of mint germplasm. In: Final Report on "Collection, characterization and evaluation of available mint (Mentha sp.) germplasm in Bangladesh" Project. Department of Horticulture, BSMRAU, Gazipur. September 2013. Ministry of Science and Technology, Dhaka. pp: 10-17.

Husain, S., Jain, A., Kothari, S. L.(2007). Phenylacetic acid improves bud elongation and *In vitro* plant regeneration efficiency in *Mentha sp* .Plant Cell Reports. **19**: 64-68.

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

Kane, M. (2014). Plant Micropropagation. Environmental Horticulture Department, University of Florida. USA.

Kehie, M., Kumaria, S., & Tandon, P. (2012). *In vitro* plantlet regeneration from nodal segments and shoot tips of Mint. Jacq. cv. Naga King Mint 3 Biotech. **2**: 31-35.

Kiran., lee., and Sharma. (2004). Hormones in tissue culture and micropropagation. In: Davies PJ ed. Plant hormones: physiology, biochemistry and molecular biology. Dordrecht: Kluwer. pp. 774-796.

Kulkarni, V. P. (2005). *In vitro* studies in mint (*Mentha sp*) Indian Journal of Genetics. **65(4**): 329-330.

Kyesmu, P.M., C.P.E. Omaliko, and A. Maduekwe, (2004). Basic Facts on Plant Tissue Culture: A Guide to Plant Tissue Culture Practice. Dolio-B Press, Abuja, Nigeria.

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 Mint varieties adapted to eastern and Southern Africa. *J. Biotech*. **9(20)**:2866- 2871.

Manik, S. R., G. M. Yatoo, Z. Ahmad and V. N. Nathar. (2012). Direct organogenesis of *Mentha spicata L*. from shoot tip, nodal and sucker explants. *J. Agril. Tech*. **8**: 663-669

Mathew, S.K., S. Millam, I. Hein. and G.J. Bryan. (2010). Cloning and molecular characterization of a potato SERK gene transcriptionally induced during initiation of somatic embryogenesis. *Planta.* **2(2)**:319-330.

Mathew, J. B. and Pious Thomas, (2002). Influence of ammonium to nitrate nitrogen ratioin different basal media on callusing response of Mint. Indian Journal of Experimental Biology. **37**: 314-316.

Nualbunruang, P. and Chidburee, A. (2004). Effect of auxins on *in vitro* rooting and effect of different growth media on growth of Mint RMUTP Research Journal Special Issue. The 4th Rajamangala University of Technology International Conference.**23(4):**344-378.

Pandya, H. A. and O. P. Saxena. (2000). Role of PGRs on tissue culture raised plantlets of Mint. Role of plant growth regulators and plant biotechnology to improve growth and productivity of plants, Botany Department, Gujarat University, Ahmedabad, India.

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

Raj, V. K. and Sankara Rao, K. (2015). Transformation of Mint variable response of transformants to leaf spot disease. *Plant Sci.***160**: 889-898.

Rama, 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.

Rajam, S. P. N. (2006). The role of endogenous auxin in root initiation. Plant Growth Regul. **13(1):** 77-84.

Reed, B. M. (1999). *In vitro* storage conditions for mint germplasm. *Hort. Sci.* **34:** 350-352.

Rizwan and Ahmed, K. (2013). Evaluation of certain insecticides for the control of pest complex on Mint in Andhra Pradesh. Pesticides. **19**: 41-44.

Ruseva, R. (1999). *In vitro* cultivation of mint (M. piperita) with high multiplication rate. *Plant Sci*. **36**: 201–203.

Safaeikhorram, M.S., S. Jafarneha and S. Khosroshahi. (2008). The world's most important medicinal plants. Compilation Eric von Ben Vick, Michael Vynk. Iran Releases Green Farm Training Complex. **3(1)**: 422.

Satner, K., & Sharma, G.J. (2016). *In vitro* plant regeneration in six cultivars of *Mentha spp*. using different explants. *Biol. Plantarum*. **52**:141–145.

Sanatombi, K., and Sharma, G.J., (2007). Micropropagation of *Mentha spicata* using axillary shoot explants, Scientia Hort., 113, 9699.Agrawal,s.,Chandra,N., and Kothari,S.L.,.1988, Shoot-tip culture of Mint for micropropagation. *Curr. Sci*. **57**: 1347-1349.

Senthil, K and M. Kamaraj. (2012). Direct shoot regeneration from inter nodal explants of *Mentha viridis* L. *Intl. J. Pharmaceutical Sci. Res*. **3**: 1101-1103.

Sharma, M.M., Dhingra, M., Dave, A. and Batra, A. (2012). Plant regeneration and stimulation of *in vitro* flowering in Eruca sativa Mill. African Journal of Biotechnology. **11(31)** : 7906-7911.

Subhash. V. K. and Sankara Rao, K. (2015). Regeneration of Mint with different hormonal doses. *Plant Sci.* **160** : 889-898.

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.

Stewart, P., and Rajam, M.V., (2007). Genotype, explant, and position effects on organogenesis and embryogenesis in Mint. *Jourl. Exptl. Bo.* **46**: 135-141.

Sujana, P. and C. V. Naidu. (2011). High frequency rapid plant regeneration from shoot tip and nodal explants of Mentha piperita L.- an important multipurpose medicinal plant. *J. Phytol.* **3**: 9-13

Sujana, P. and C.V. Naidu. (2011). High frequency rapid plant regeneration from shoot tip and nodal explants of Mentha piperita L.- an important multipurpose medicinal plant. *J. Phytology*. **3(5)** : 9-13.

Sunandakumari C, Martin KP, Chithra M, Sini S, Madhusoodanan PV (2004). Rapid axillary bud proliferation and ex vitro rooting of herbal spice, *Mentha piperita* L. *Ind. J. Biotech*. **3**: 108-112.

Szasz A., mahashi. H., Viki. H. (2005) Vanilloid receptors in health and disease. *Amer. J. Clin Pathol*. **118**: 110-121.

Tuly, J. A., M. A. Hoque and M. M. Hossain. (2015). *In vitro* shoot regeneration of mint (*Mentha sp*.) using different explants and genotypes. *Ann. Bangladesh Agric*. 19: 53-64

Van. L.B. (2009). Plant tissue culture (overview) Part II. Plant Biotechnology. University of Science. Vietnam Open Course Ware.

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

Vikram. M., Dhingra, M., Dave, A. and Batra, A. (2015). Plant regeneration and stimulation of *in vitro* flowering in *Mentha piperita L.*. *African J. of Biot.* **11(31)**: 7906-7911.

Yanpaisan, W., King, N.J. and Doran, P.M. (1999). Flow cytometry of plant cells with in large-scale bioprocessing. *Biotech Advance*. **17**: 3-27

Yapo, E.S., Kouakou, T.H., Kone, M., Kouadio, J.Y., Kouam , P. and Merillon, J.M.(2011). Regeneration of Mint plant through somatic embryogenesis. *J. plant Bioch. and Biot.* **20(2)**:194-204.

Zhang, T., Cao, Z.Y. and Wang, X.Y. (2005). Induction of somatic embryogenesis and plant regeneration from cotyledon and hypocotyl explants of Mint. *In vitro* Cellular & Developmental Biology Plant. **41(5)**: 655-657.

Zhu, N., Ellis, J. and Pelletier, G., (2014). *In vitro* cultivation of mint (*M. spicata*) with high multiplication rate. *J. of Biot.* **315**: 111-216.

APPENDICES

Appendix I. Analysis of variance (ANOVA) of effect of different concentration of BA on days to shoot initiation, percent of shoot and number of leaves at different DAI

Source of variance	d.f	Days to shoot initiation	Percent (%) Shoot Initiation	Number of leaves		
				21 DAI	28 DAI	42 DAI
Treatment	3	10.562	395.833	7.500	12.166	37.729
Error	9	0.340	8.333	0.166	0.166	0.562
Total	15					

**= Significant at 1% level of Probability.

Appendix II. Analysis of variance (ANOVA) of effect of different concentration of BA on number of shoot at different DAI

Source of variance	d.f	Number of shoot		
		21 DAI	28 DAI	42 DAI
Treatment	3	18.500	65.729	119.562
Error	9	0.444	0.284	0.229
Total	15			

**= Significant at 1% level of Probability.

Appendix III. Analysis of variance (ANOVA) of effect of different concentration of BA on length of shoot at different DAI

Source of variance	d.f	length of shoot		
		21 DAI	28 DAI	42 DAI
Treatment	3	6.083	15.390	20.182
Error	9	0.138	0.196	0.335
Total	15			

**= Significant at 1% level of Probability.

Appendix IV. Analysis of variance (ANOVA) of effect of different concentration of BA on length of leaves at different DAI

Source of variance	d.f	Number of leaves		
		21 DAI	28 DAI	42 DAI
Treatment	3	1.729	4.500	11.557
Error	9	0.076	0.208	0.2934
Total	15			

**= Significant at 1% level of Probability.

Appendix V. Analysis of variance (ANOVA) of effect of different concentration of IBA on days to root initiation, percent of root and number of roots at different DAI

Source of variance	d.f	Days to root initiation	Percent (%) root Initiation	Number of root		
				21 DAI	28 DAI	42 DAI
Treatment	3	13.416	603.583	16.1666	46.395	83.000
Error	9	0.472	9.972	0.111	0.451	0.833
Total	15					

**= Significant at 1% level of Probability.

Appendix VI. Analysis of variance (ANOVA) of effect of different concentration of IBA on length of root at different DAI

Source of variance	d.f	length of root		
		21 DAI	28 DAI	42 DAI
Treatment	3	1.729	4.500	11.557
Error	9	0.076	0.208	0.293
Total	15			

**= Significant at 1% level of Probability.

Appendix VII. Analysis of variance (ANOVA) of combined effect of different concentration of BA and IBA on days to shoot initiation, percent of shoot initiation and number of shoot at different DAI

Source of variance	d.f	Days to shoot initiation	Percent (%) Shoot Initiation	Number of Shoot		
				21 DAI	28 DAI	42 DAI
Treatment	9	1.074	5.910	18.500	65.729	119.562
Error	18	0.241	4.000	0.444	0.284	0.229
Total	47					

**= Significant at 1% level of Probability.

Appendix VIII. Analysis of variance (ANOVA) of combined effect of different concentration of BA and IBA on number of leaves at different DAI

Source of variance	d.f	Number of leaves		
		21 DAI	28 DAI	42 DAI
Treatment	9	0.407	0.453	0.981
Error	30	0.384	0.727	0.694
Total	47			

**= Significant at 1% level of Probability.

Appendix IX. Analysis of variance (ANOVA) of combined effect of different concentration of BA and IBA on length of shoot at different DAI

Source of variance	d.f	Length of shoot		
		21 DAI	28 DAI	42 DAI
Treatment	9	0.325	0.669	0.852
Error	30	0.099	0.142	0.052
Total	47			

**= Significant at 1% level of Probability.

Appendix X. Analysis of variance (ANOVA) of combined effect of different concentration of BA and IBA on days to root initiation, percent of root and number of roots at different DAI

Source of variance	d.f	Days to shoot initiation	Percent (%) root Initiation	Number of root		
				21 DAI	28 DAI	42 DAI
Treatment	9	0.2616	3.30	0.231	0.428	0.336
Error	30	0.515	10.66	0.234	0.239	0.390
Total	47					

**= Significant at 1% level of Probability.

Appendix XI. Analysis of variance (ANOVA) of combined effect of different concentration of BA and IBA on length of root at different DAI

Source of variance	d.f	length of root		
		21 DAI	28 DAI	42 DAI
Treatment	9	0.079	0.347	0.567
Error	30	0.056	0.074	0.189
Total	47			

**= Significant at 1% level of Probability.

Appendix XII. Analysis of variance (ANOVA) of effect of BA and IBA on Days to shoot and number of shoot at different DAI

Source of variance	d.f	Days to shoot initiation	Percent (%) Shoot Initiation	Number of Shoot		
				21 DAI	28 DAI	42 DAI
Treatment	9	0.928	5.91	0.083	0.262	0.111
Error	30	0.108	4.00	0.209	0.539	0.376
Total	47					

**= Significant at 1% level of Probability.