MICROPROPAGATION OF BLACK PEPPER (*Piper nigrum* L.)

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CERTIFICATE

This is to certify that the thesis entitled, ***''MICROPROPAGATION OF BLACK PEPPER (Piper nigrum L.)''*** *submitted to the Faculty of Agriculture, Sher-e-Bangla Agricultural University, Dhaka, in partial fulfillment of the requirements for the degree of* ***MASTER OF SCIENCE (MS) IN BIOTECHNOLOGY****, embodies the results of a piece of bona fide researchwork carried out by* ***TANIA AFROZ,*** *Registration No.* ***14-06113*** *under my supervision and guidance. No part of this thesis has been submitted for any other degree or diploma.*

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

Dated:

Dhaka, Bangladesh

(Prof. Dr. Md. Ekramul Hoque)
Supervisor

ABBREVIATIONS AND ACRONYMS

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

ABBREVIATIONS AND ACRONYMS

Abbreviation	**Full Word**
mg/L	: Milligram per litre
Mm	: 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

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The Author

TABLE OF CONTENTS

TABLE OF CONTENTS

TABLE OF CONTENTS

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF APPENDICES

LIST OF APPENDICES

LIST OF APPENDICES

MICROPROPAGATION OF BLACK PEPPER (*Piper nigrum* L.)

ABSTRACT

The present study was undertaken in the Biotechnology Laboratory of the Department of Biotechnology, Sher-e-Bangla Agricultural University, Sher-e-Bangla Nagar, Dhaka-1207, during the period from January to September, 2020 to evaluate the effect of Benzyl adenine (BA) Indole-3-acetic acid (IBA) on *in vitro* regeneration of Black pepper. The nodal segments of Black pepper were used as explants which were inoculated in MS media supplemented with different conc. (0.5, 1.0, 1.5 and 2.0 mg/L) of BA and IBA (0.5, 1.0, 1.5 and 2.0 mg/L) either alone or in combination of BA (1.0, 1.5, 2.0 and 2.5 mg/L) and IBA (1.0, 1.5, 2.0 and 2.5 mg/L). The experiments were arranged in Completely Randomized Design (CRD) with five replications. The maximum shoot induction was obtained with 2.0 mg/L BA.The highest number of leaves were recorded in the treatment 2.0 mg/L BA at 8 WAI. The maximum number of root was regenerated in the treatment 2.0 mg/L IBA. The combination of both the hormone 2.0 mg/L BA + 2.0 mg/L IBA showed best result for shoot induction, shoot length and also for total number of leaf. Maximum percentage of root induction and root length also found in the same treatment. Regenerated plantlets survive 46.67% in shade house and 57.14% in open atmosphere. Therefore, an efficient protocol has been developed for *in vitro* regeneration of Black pepper which can be used for large scale production of Black pepper.

CHAPTER I

INTRODUCTION

Black pepper (*Piper nigrum* L.) is a flowering vine in the family Piperaceae, cultivated for its fruit, known as a peppercorn. It is usually dried and used as a spice and seasoning. When fresh and fully mature, the fruit is about 5 mm (0.20 inch) in diameter and contains a single seed. Peppercorns and the ground pepper derived from them may be described simply as pepper, or more precisely as black pepper (cooked and dried unripe fruit), green pepper (dried unripe fruit), or white pepper (ripe fruit seeds) (Harrison and Paul, 2016).The word pepper derives from old English "pipor", Latin "piper",and Sanskrit "pippali" for "long pepper". In the 16th century, people began using pepper to also mean the unrelated new world chili pepper (genus *Capsicum*). In Bangladesh it is mostly known as "Golmorich". It is native to India, Indonesia, Malaysia, South America and West Indies. But it isalso widely cultivated in the tropical regions. It is christened as the 'King of Spices' (Srinivasan, 2007; Mathew *et al*., 2001).

Ground, dried and cooked peppercorns have been used since antiquity, both for flavour and as a traditional medicine. Black pepper is the world's most traded spice, and is one of the most common spices added to cuisines around theworld. Its spiciness is due to the chemical compound piperine, which is a differentkind of spicy from the capsaicin characteristic of chili peppers. It is ubiquitous inthe modern world as a seasoning, and is often paired with salt and available on dining tables in shakers or mills.

The seeds and fruits are mostly used. The preferred mode of preparation is in powdered form, pills or tablets, and paste. *Piper nigrum* and its bioactive compounds were also found to possess important pharmacological properties. Antimicrobial activity was recorded against a wide range of pathogens via

inhibition of biofilm, bacterial efflux pumps, bacterial swarming, and swimming motilities. Studies also reported its antioxidant effects against a series of reactive oxygen and nitrogen species including the scavenging of superoxide anion, hydrogen peroxide, nitric oxide. Improvement of antioxidant enzymes *in vivo* has also been reported. *Piper nigrum* also exhibited anticancer effect against anumber of cell lines from breast, colon, cervical, and prostate through different mechanisms including cytotoxicity, apoptosis, autophagy, and interference with signaling pathways. Its antidiabetic property has also been confirmed in vivo as well as hypolipidemic activity as evidenced by decrease in the level of cholesterol, triglycerides, and low-density lipoprotein and increase in high-density lipoprotein. *Piper nigrum* also has anti-inflammatory, analgesic, anticonvulsant, and neuroprotective effects. The major bioactive compound identified in *Piper nigrum* is piperine although other compounds are also present including piperic acid, piperlonguminine, pellitorine, piperolein B, piperamide and piperettine which also showed biological potency (Takoore *et al*., 2019). It is an excellent source of manganese, a very good source of iron and vitamin K, and a good source of dietary fibre.

Black pepper is the most preciousand valuable form of spices in the world. It is the 3rd most added ingredient in food among the wide range of spices. A wide variety of black pepper is traded atan international level. India is one of the top five exporters of black pepper, along with Vietnam, Indonesia, Brazil and Malaysia. Black pepper, being the eldest spice in the world, is seen both as a taste enhancer and as a medicine.

Black pepper grows in many parts of Bangladesh, particularly in hilly terrain. But there is no statistics information available in our country about area and production of Black pepper. Every year huge amount of black pepper is used in our country as a spiceand medicinal purpose. Most of them are imported from abroad.

Black pepper can be propagated by cuttings, layering and grafting. Seed propagation often results in genetic variation due to formation of recombinants while other methods of black pepper propagation are slow and time consuming. So, there is a need to introduce efficient methods for faster propagation of black pepper.

In this context, plant tissue culture is the most efficient and reliable method for rapid and mass scale production of disease free, genetically stable and identical progeny (Hussain *et al*., 2011). Tissue culture techniques have taken significant part in clonal propagation, conservation of germplasm and plant improvement in black pepper (Cantelmo *et al*., 2013; Sajc *et al*., 2000). If a technique could be evolved, it could facilitate rapid clonal multiplication of planting materials in a comparatively short period of time in Bangladesh context.

Plant hormones (also known as phytohormones) are signal molecules, produced within plants, that occur in extremely low concentrations. Plant hormones control all aspects of plant growth and development, from embryogenesis (Mendez-Hernandez *et al*., 2019), the regulation of organ size, pathogen defense (Shigenaga et al., 2016; Burger *et al*., 2019), stress tolerance (Ku *et al*., 2018; Ullah *et al*., 2018) and through to reproductive development (Pierre-Jerome *et al*., 2018). Different hormones can be sorted into different classes, depending ontheir chemical structures. Within each class of hormone, chemical structures canvary, but all members of the same class have similar physiological effects. Initial research into plant hormones identified five major classes: auxins,cytokinins, ethylene, gibberellins and abscisic acid (Thomas *et al*., 1979). Among them, auxins are compounds that positively influence cell enlargement, bud formation, and root initiation. Auxins, especially indole-3-acetic acid (IAA), 1-naphthaleneacetic acid (NAA) and indole-3-butyric acid (IBA) are commonly applied to stimulate root growth. Cytokinins are a group of chemicals that influence cell division andshoot formation.

Considering the above facts, the present investigation has been undertaken to find out the performance of different hormones (BA and IBA) with the optimum concentration for *in vitro* plantlets regeneration of Black pepper. Hence, the experiment was designed to fulfil the following objectives.

i. To Assess the hormonal effect on *in vitro* regeneration of Black pepper

ii. To identify the best hormonal concentration for *in vitro* regeneration of Black pepper

iii. To study the micropropagation of Black pepper within a short period of time and

iv. To establish a protocol for micropropagation of Black pepper

CHAPTER II

REVIEW OF LITERATURE

The present investigation involved micropropagation of *Piper nigrum*. Plant regeneration from culture media of shoot tips and nodal segments followed by genetic stability of plantlets seems to be meager. However, information available in these aspects have been reviewed and presented in these sections:

2.1 Concept of plant tissue culture

Plant tissue culture, also referred to as cell, *in vitro*, axenic, or sterile culture, is an important tool in both basic and applied studies, as well as in commercial application (Thorpe *et al*., 1990). Plant tissue culture is the aseptic culture of cells, tissues, organs and their components under defined physical and chemical conditions *in vitro*. The theoretical basis for plant tissue culture was proposed by Gottlieb Haberlandt in his address to the German Academy of Science in 1902 on his experiments on the culture of single cells (Haberlandt, 1902).

Black pepper is conventionally propagated through cuttings with 2-6 nodes for nursery and field plantations. Among the major weakness responsible for low productivity of black pepper, non-availability of healthy planting materials and crop losses due to biotic and abiotic stresses are of foremost importance (Sharma and Kalloo, 2004). In this context, plant tissue culture is the most efficient and reliable method for rapid and mass scale production of disease free, genetically stable and identical progeny of black pepper throughout the year (Hu and Wang, 1983).

2.2 Sterilization of explant

In *Aconitum heterophyllum* (Atish), 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).

In groundnut, $HgCl_2$ 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).

In cultivated *Piper* species, explants were subjected to 50% ethanol for 30 seconds, washed with distilled water and then placed in 0.1% mercuric chloride for 2 minute and again washed with distilled water in laminar airflow cabinet (Bhat *et al*., 1995).

In Banana, ascorbic acid is used as surface sterilant for reducing oxidization of explant (Khatun, 2014).

In Black Pepper, the explants were given a quick rinse in 70% ethyl alcohol for 45 seconds, then immersion in 0.10% mercuric hypochloride in water for 1 min, followed by several rinses with sterile-distilled water (Ahmad *et al*., 2011).

In Black Pepper, pre-sterilized explant cuttings (6-7mm) were taken and cut into segments of 4-5 mm each in length. Explants harvested were first washed with running tap water and then treated with household detergent for five minutes. This was followed by second washing with tap water to remove all traces of detergent. The explants were then treated with 0.1% mercuric chloride for one minute. To remove all the traces of mercuric chloride, the explants were washedthree times with sterilized distilled water (Hussain *et al*., 2011).

In Black Pepper, explants were kept under running tap water for 30 minutes, then washed with mild detergent and washed with tap water. After this, the explants were taken into laminar airflow cabinet, added 70% alcohol for 1 minute then rinsed off with sterile distilled water. Finally added 0.1% $HgCl_2$ (w/v) for 5 minutes for nodal explants and 3 minutes for leaf explants with continuous stirring followed by washing 3 times with sterile distilled water (Khan *et al*., 2016).

2.3. Multiple shoot induction

Hussain *et al*. (2011) reported that in Black Pepper, when 0.5 mg/L of BA was used in MS medium, 100% shoot formation was obtained after 14 days of callus inoculation. Good shoot formation was noticed at 1.0 and 1.5 mg/L and fair with 2.0 mg/l of BA.

Anand and Rao (2000) who observed that in *Piper barberi gamble*, the shoot tip and nodal explants failed to stimulate shoot regeneration on cytokinin-free medium. They also reported that increasing the concentration of BA from 0 to 6 μM enhanced regeneration but further increase in the concentrations of BA and kinetin suppressed number of shoot formation.

Kadam *et al.* (2020) reported that in Black Pepper, the best multiple shoot initiation was observed on the media combination of the media (MS + 4.5 mg/L BAP + 1.0 mg/L IAA) showed maximum shoot induction.

Mathews *et al*. (1984) studied that in Black Pepper, the regeneration of multiple shoot buds occured in shoot tip cultures of seedlings on MS medium supplemented with IAA and BA (1.0 mg/L each).

Umadevi *et al.* (2015) examined that in Black Pepper, the meristem extension was achieved in liquid medium containing Murashige and Skoog with 0.1 mg/L kinetin and 0.5 mg/L GA_3, subsequent direct shoot induction in half strength MS media with 3 mg/L BA and 1 mg/L IAA followed by shoot growth and development in half strength MS +0.5 mg/L Indole butyric acid. The meristems of 2mm resulted in shoot induction and proliferation via direct organogenesis.

Rubluo and Barroso (1992) who reported that in Pepper, BA and IAA gave considerably ineffective in shoot formation.

Khan *et al.* (2017) reported that in Black Pepper, the best response towards multiple shoot formation was obtained on MS with 1.0 mg/L of BAP and 1.0

mg/L IAA (60%) where mean number of shoots per explant was 4. Callus and shoot regeneration were encouraged from leaf portions on Murashige and Skoog (MS) medium augmented with varied concentrations of plant growth regulators. A higher callus production (90 %) was observed in explants incubated on MS medium incorporated with 1.0 mg/L

Ahmad *et al.* (2013) studied that in Black Pepper, a callogenic response of 85 % was also recorded for 1.0 mg/L BA in combination with 0.25 mg/L α-naphthalene acetic acid (NAA) and 0.25 mg/L 2,4-dichlorophenoxyacetic acid or 0.5 mg/L indole butyric acid (IBA) along with 0.25 mg/L NAA and indole acetic acid. Subsequent sub-culturing of callus after 4 weeks of culture onto MS medium supplemented with 1.5 mg/L thiodiazoran or 1.5 mg/L IBA induced 100 % shoot response.

Ahmad *et al*. (2011) examined that in Black Pepper, callogenesis was induced from petiole explants of potted plants. It was incubated on murashige and skoog (MS) medium supplemented with different concentrations of several phytohormones (PGRs). The best callus induction (85%) was observed for MS-medium supplemented with 0.5 mg/L 6 - benzyladenine (BA) after 4 weeks of culture. Subsequent transfer of callus to MS medium containing similar PGRs induced shoot regeneration. Highest shoot regeneration (92%) was recorded for 0.5 mg/L BA after 5 weeks of transfer. Furthermore, 8.1 shoots/explant were recorded for 0.5 mg/L BA. Addition of 2.0 mg/L of indole-3-butyric acid (IBA) produced 5.1 cm long shoots with 85%.

Maju *et al.* (2012) reported that in Black Pepper, direct induction of shoots was from bulged portion of shoot tip and nodal segments. Shoot tip and nodal segments cultured on Schenk and Hildebrand (SH) and Murashige and Skoog (MS) media containing various combinations of cytokinins (0.5-4 mg/L BA and 0.05-0.5 mg/L TDZ) and auxins (0.2-1 mg/L IAA, 0.05-0.5 mg/L NAA, 0.5-6 mg/L Kin, 0.2-0.6 mg/L 2,4-D). The best shoot proliferation (18±1.7

shoots/explant) response was observed in SH medium with a combination of 2.5 mg/L BA and 0.5 mg/L IAA.

Bhat *et al.* (1995) studied the morphogenetic potential of root, leaf, node and internode explants of 3 cultivated *Piper* species. It was investigated to develop a reliable plant regeneration protocol. *P. longum* (Pipli) was the most responsive followed by *P. betle* (Betel vine) and *P. nigrum* (Black pepper). In *P. longum* the highest number of shoot buds was produced on root explants followed by node, internode and leaf expiants. In *P. betle* and *P. nigrum* adventitious shoot buds differentiated only from internodal and nodal ring regions, respectively. Histological examination in *P. longum* showed that adventitious shoot buds originate directly from the cortical cells of the root and the internode without an intervening callus phase. Benzyladenine was superior to kinetin for shoot induction and its optimum concentrations for *P. longum*, *P. betle* and *P. nigrum* were 1–2, 10 and 10 μM, respectively. rooting was achieved in B_5 medium containing 0.5 μM benzyladenine and 1 μM indoleacetic acid, respectively.

Nair *et al.* (2003) examined that in Black Pepper, explants were cultured on growth regulator – free solid SH (Schenk and Hildebrandt) medium maintained in the dark. The first somatic embryos developing directly from the explant tissue were noticed after 60 days of culture. Somatic embryos originated from a ring-like tissue on the micropylar region of the seeds. Sucrose concentration of the medium was found to be crucial for the induction of somatic embryos, and 30 gm/L was found to be the optimum. Maturation and germination of somatic embryos were achieved on the same medium. Suspension culture enhanced the process of maturation and germination.

Rahman *et al*. (2007) reported that immature zygotic embryos of *Piper nigrum* L. were cultured in different concentrations and combination of auxins and cytokinins. Callus formation was highest (92.2%) when the explants were

cultured on Murashige and Skoog (MS) medium supplemented with 2.0 mg/L IAA and 0.5 mg/L kinetin. Calli were transferred to same basal medium containing 1.5 mg/L BAP and 0.5 mg/L NAA, where they developed maximum number of adventitious buds within three weeks of culture.

Thuyen *et al.* (2005) studied that the shoot initiation and the establishment. The optimal concentration of black pepper growth was BA 2.0 mg/L, IBA 0.5 mg/L. The high concentrations of BA (5-10 mg/L) had no effect on the shoot regeneration from callus and the development of lateral branches. The MS medium containing BA 5.0 mg/L, Kin 0.5 mg/L and IBA 0.5 mg/L was the best medium for the subsequent growth and the shoot regeneration from callus.

2.4 Induction of root

Hussain *et al*. (2011) studied that in Black Pepper, excellent rooting was shown by 1.5 mg/L IBA on MS medium in all cultures within 8 days. The plantlets formed were rooted best on 1.5 mg/l IBA.

Azad *et al*. (2003) observed that in Malabar Nut, different concentrations of BA (cytokinin) ranging from 0.5–3.0 mg/L on MS medium produced no roots from the regenerated shoots because the process of *in vitro* root initiation, development and elongation normally require medium containing Auxin.

Mathews *et al.* (1984) examined that in Black Pepper, *in vitro* regenerated shoots rooted when induced to half strength MS medium supplanted with 0.2 mg/L of NAA.

Umadevi *et al.* (2015) reported that in Black Pepper, successful rooting of meristem derived shoots was achieved in half strength WPM (Woody Plant Medium) with 3 mg/L BA and 1 mg/L Kinetin.

Khan *et al*. (2017) reported that in Black Pepper, healthy roots developed from the base of the 80% shoots cultured on half strength of MS medium containing 1.5 mg/L IBA.

Maju *et al*. (2012) studied at in Black Pepper, the resulting shoots were subsequently rooted on half strength SH medium containing 0.5 mg/L IBA within four weeks with 90% success.

Bhat *et al.* (1995) examined that in different *Piper* species, rooting was achieved in B5 medium containing 1 μM indole acetic acid.

Rahman *et al*. (2007) reported that in Black Pepper, the regenerated shoots were elongated on MS medium containing 1.5 mg/L BAP and 0.5 mg/L NAA and subsequently rooted in half strength MS medium with 1.5 mg/L IBA.

Thyuen *et al.* (2005) observed that in Black Pepper, the shoots were rooted on half strength MS medium containing NAA 0.1-0.2 mg/L.

2.5 Hardening and establishment of the regenerated plant

In Black Pepper, rooted plantlets were removed from the medium and transferred to pots containing compost, sand, and soil media in 1:1:1 ratio. The plants were covered with transparent polythene bags for two weeks for acclimatization (Philip *et al.,* 1992).

Polythene covers were gradually removed over a period of 2 weeks in various explants of cultivated *Piper* species. (Bhat *et al.,* 1995).

Hussian *et al.* (2011) reported that the rooted plants were transferred to green house for four weeks and then successfully test planted in the fields. Black pepper being a vine needs support for climbing. Four different tree species were selected to provide support for vines i.e., *Eucalyptus camaldulensis*, *Grevillea robusta*, *Pinus roxburghii* and *Phyllanthus emblica*. The vine grew best on Silver Oak (*Grevillea robusta*) tree round the year. Black pepper vine is frost tender. The

vines growing in green house during winter season had better health than those growing open in air.

Anand and Rao (2000) reported that the use of growth chamber under high humidity conditions for acclimatization of Black pepper plants.

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 January to September, 2020.

3.2 Experimental materials

3.2.1 Source of materials

The planting materials of *Piper nigrum* (Black pepper) were collected from the different nurseries of Agargaon, Sher-e-Bangla Nagar, Dhaka-1207.

3.2.2 Plant materials

The nodal segments of *Piper nigrum* (Black pepper) were used asexperimental materials in the present research work. Shoot with young leaves were collected from the Black pepper 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.

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.

3.2.4 Glassware

In all the experiments the borosil glassware was used given priority. The glassware was 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 was 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 (Appendix I). 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 I (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 II (Micronutrients)

The stock solution of micronutrients was made up to 100 times the finalstrength 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 III (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 distilledwater. Finally, the stock solution was filtered and stored in a refrigerator at 4^0C.

3.3.4. Stock solution IV (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 0^0C. Myo-inositol was used directly at the time of media preparation.

3.3.5. Hormone stock solution

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 (0.5, 1.0, 1.5, 2.0 mg/L) for shoot induction

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

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 100% ethyl alcohol. Hormone (solute) Solvents used BA 1N NaOH and IBA 100% ethyl alcohol. In present experiment, the stock solution of hormones was 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 100% 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 ± 10^0C for use up to three months.

3.5 Sub- experiments

Four 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.

3.5.1 Sub-experiment 1.

Effect of BA on *in vitro* shoot induction potentiality in Black pepper

Four levels of BA (0.5, 1.0, 1.5 and 2.0 mg/L) and control (0.0 mg/L) treatments were used. The experiments were arranged in Completely Randomized Block (CRD) with five replications.

3.5.2 Sub-experiment 2

Effect of IBA on root induction potentiality of in vitro regeneration in Black pepper

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 procedure was entirely following sub-experiment 1.

3.5.3 Sub-experiment 3

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

In this sub-experiment, four levels of IBA (1.0, 1.5, 2.0 and 2.5 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 was also practiced. The combine treatments were as follows:

1. T1 = BA 1.0 mg/L + 1.0 mg/L IBA

2. T2 = BA 1.0 mg/L + 1.5 mg/L IBA

3. T3 = BA 1.0 mg/L + 2.0 mg/L IBA

4. T4 = BA 1.0 mg/L + 2.5 mg/L IBA

5. T5 = BA 1.5 mg/L + 1.0 mg/L IBA

6. T6 = BA 1.5 mg/L + 1.5 mg/L IBA

7. T7 = BA 1.5 mg/L+ 2.0 mg/L IBA

8. T8 = BA 1.5 mg/L + 2.5 mg/L IBA

9. T9 = BA 2.0 mg/L + 1.0 mg/L IBA

10. T10 = BA 2.0 mg/L + 1.5 mg/L IBA

11. T11 = BA 2.0 mg/L + 2.0 mg/L IBA

12. T12 = BA 2.0 mg/L + 2.5 mg/L IBA

13. T13 = BA 2.5 mg/L + 1.0 mg/L IBA

14. T14 = BA 2.5 mg/L + 1.5 mg/L IBA

15. T15 = BA 2.5 mg/L + 2.0 mg/L IBA

16. T16 = BA 2.5 mg/L + 2.5 mg/L IBA

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

3.1.1 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.

3.2 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.

3.6 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/cm^2 (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.

3.7 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.

3.8 Preparation of explants and sterilization

To prepare explants following steps were followed:

I. The explants (nodal segments and shoot tips) were washed thoroughly under running tap water.

II. The explants are soaked with trix for 10 minutes.

III. Then washed with distlled water for several times.

IV. After that explants were put into 100 mg/L Ascorbic acid solution for 20 minutes.

V. After 20 minutes the explants were removed from that solution and washed several times with distilled water in laminar Air Flow Cabinet.

VI. Then sterilized with 70% ethanol for 1 min and after 1 minute washed with distilled water.

VII. Again the explants were sterilized with 0.2- 0.5% $HgCl_2$ with few drops of Tween 20 solution for 5 minutes.

VIII. Then the explants were washed with distlled water at least 3 times.

IX. The final size of the explants were 0.5-1 cm.

X. Finally the explants were ready to transfer in the culture vessel carefully.

3.9 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 in LAFC. Here the explants were further trimmed, and extra outer leaves were removed with sterile scalpel blade to make suitable size. The mouthof 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 were 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

3.10 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±1º 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.

3.11 Sub-culturing and maintaining of proliferating shoots

Initial sub-culturing was done after 30 to 40 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 ofsterile 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 after2nd 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.

3.12 Rooting of *in vitro* induced shoots

Shoots (3.0 cm) derived from shoot bunches were excised and rooted on medium consisting of MS basal medium supplemented with IBA at each (0.5, 1.0, 1.5 and 2.0 mg/L). All the media used in this study were supplemented with3% (w/v) sucrose, solidified with 0.8 % (w/v) agar and the pH was adjusted to 5.8± 0.1 before autoclaving at 121^0C and 1.06 kg/cm^2 (15 PSI) for 15 min.

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. Five replicates (single shoot per culture bottle) were used per treatment. Data were recorded after 3, 5 and 8 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 eighth 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 explant

3. Length of shoot (cm)

4. No. of leaves per explant

5. Days to root induction

6. No. of roots per explant

7. Length of root (cm)

8. Percent of explants showing shoot induction

9. Percent of explants showing root induction

3.15.1 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.

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

3.15.2 Days to shoot induction

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

3.15.3 Number of shoots per explant

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

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

3.15.4 Calculation of shoot length (cm)

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

3.15.5 Number of leaves

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

3.15.6 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.

3.15.7 Days to root induction

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

3.15.8 Number of roots/plantlet

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

$$\text{Number of root per explant} = \frac{\text{Number of roots per explant}}{\text{Number of observation}}$$

3.15.9 Length of roots

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

3.15.10 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:

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

3.16 Statistical data analysis

Data recorded for different parameters under study were statistically analysed 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 five replications. The significant difference between the pair of means was evaluated at 5% 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 shoot and root induction in Black pepper. The relative growth performance of different growth regulators has been successfully studied in the current investigation. The results obtained from the experiment were described and discussed here and the analyses of variance (ANOVA) are presented in Appendix II-XXXV.

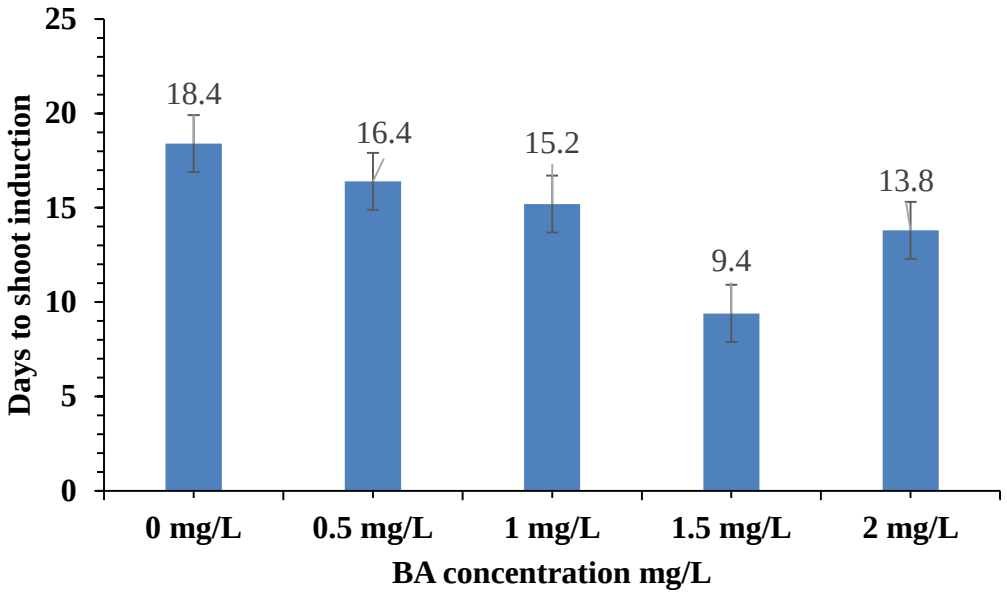
4.1 Sub-experiment 1.

Effect of BA on shoot induction potentiality in Black pepper (*Piper nigrum* L.)

This experiment was conducted under laboratory condition to evaluate the effect of different concentration of BA on shoot proliferation. The result of different concentration of BA has been presented under following headings with Figure (1-3), Plate (2-3) and Table (1-2).

4.1.1 Days to shoot induction

Significant variations were observed among different concentrations of BA on days to shoot induction. The maximum 18.4 days to shoot induction were recorded in controlled treatment followed by the treatment 0.5 mg/L BA (16.4 days) and 1.0 mg/L BA (15.2 days). On the other hand, minimum 9.4 days were noticed in 1.5 mg/L BA followed by 2.0 mg/L of BA (13.8 days) (Figure 1). Legesse *et al*. (2017) found the lowest response of number of shoots 2.0 mg/L BA while the highest response was observed in medium supplemented with 5.0 mg/L BA in Black Pepper.

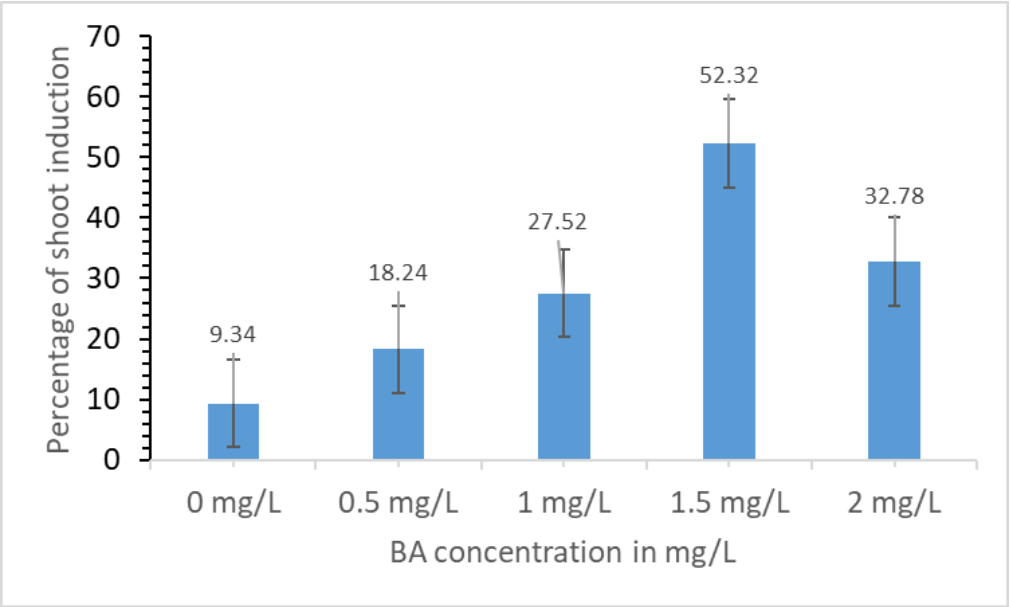


*I= Standard error bar

Figure 1. Effect of BA on days to shoot induction in Black pepper

4. 1.2 Percentage of shoot induction

The different concentrations of BA showed the significant variations on percentage of shoot induction. The treatment 1.5 mg/L BA had produced the highest frequency of shoot (52.32%), while the lowest percentage (9.34%) was in control treatment (Figure 2). On the contrary, 27.52% and 32.78% shoot induction were observed respectively from 1.0 mg/L and 2.0 mg/L BA (Figure 2). Hussain *et al.* (2011) reported that 0.5 mg/L BA in MS medium produced 100% shoot and good shoot formation was noticed at 1.0 and 1.5 mg/L in Black pepper partially contradicts with the result.



*I= Standard error bar

Figure 2. Effect of BA on percentage of shoot induction in Black pepper

4.1.3 Number of shoot per plantlet

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 (1.8, 2.6 and 3.0) at 3 WAI, 5 WAI and 8 WAI, respectively (Plate 2) was noticed from the treatment 1.5 mg/L BA. Then 0.5 mg/L, 1.0 mg/L and 2.0 mg/L of BA contained media showed (1.0, 1.2 and 2.0), (1.0, 1.6 and 2.0) and (1.2, 2.0 and 2.0) number of shoot at 3 WAI, 5 WAI and 8 WAI respectively (Table 1). Whereas shoot regeneration was not observed in control treatment at 3 WAI, 5 WAI but very incipient shoot found at 8 WAI. Soniya and Das, (2002) reported that maximum number of shoot was recorded on MS medium supplemented in 2.0 mg/L BA for *piper nigrum*. Which partially contradicts with the result. Legesse *et al*. (2017), reported that 4.0 mg/L BA was best for shoot proliferation of *Piper nigrum*. Shoot regeneration response is less in Black pepper. It might be the genetical ability of the spices crop.

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

BA mg/L	No of shoot per plant		
	3 WAI	5 WAI	8 WAI
0	0 c	0 d	1.0 c
0.5	1.0 b	1.2 cd	2.0 b
1.0	1.0 b	1.6 bc	2.0 b
1.5	1.8 a	2.6 a	3.0 a
2.0	1.2 b	2.0 b	2.0 b
CV %	28.28	27.03	15.81
LSD (0.05)	0.37	0.53	0.42

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.

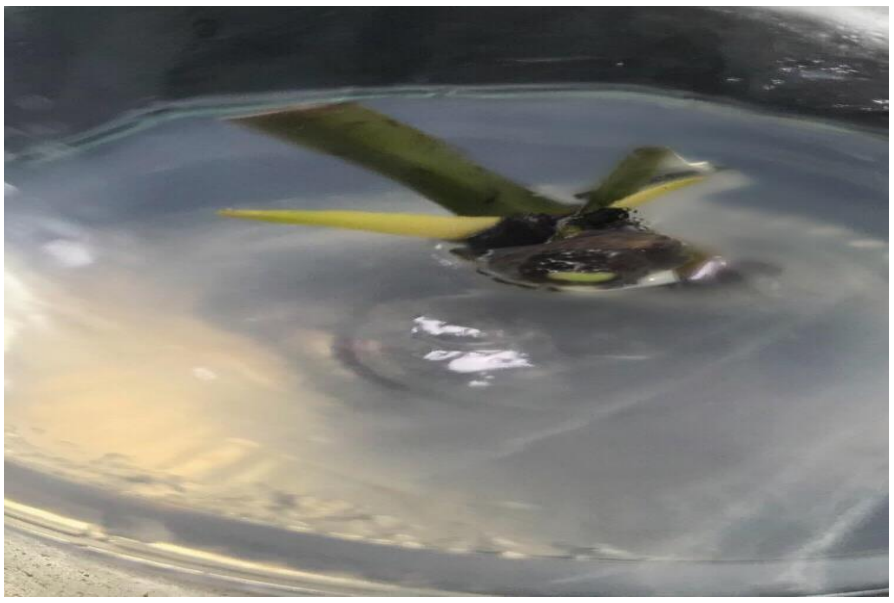
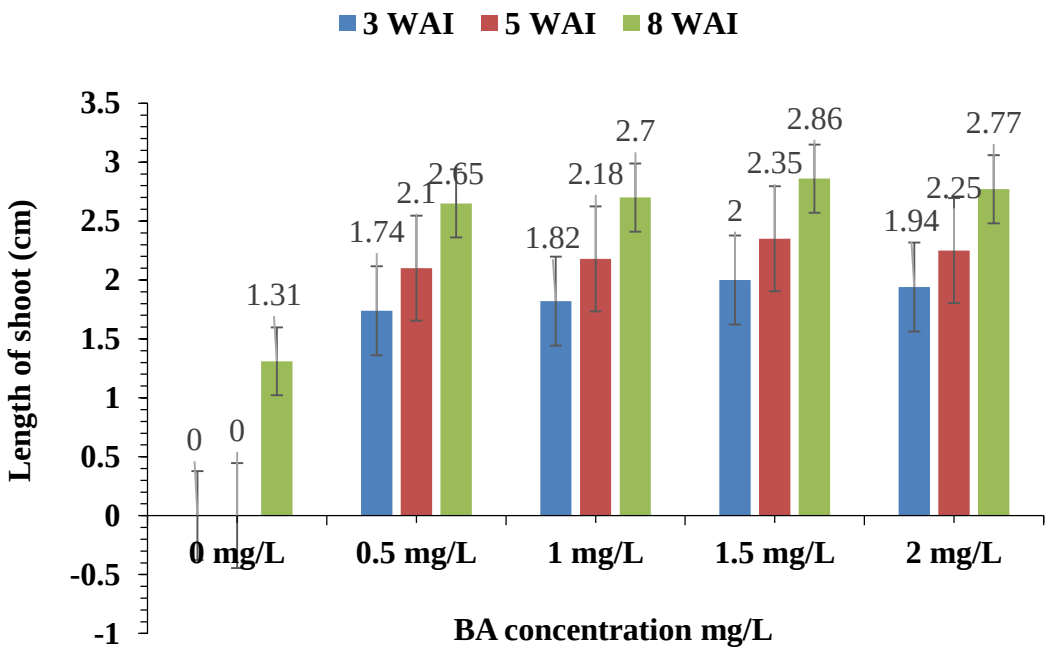


Plate 2. Number of shoot in MS medium supplemented with BA 1.5 mg/L at 8 WAI

4.1.4 Length of shoot (cm)

Significant variation of different concentrations of BA on length of shoot was found. The maximum length of shoot (2.0 cm, 2.35 cm and 2.86 cm) at 3 WAI, 5 WAI and 8 WAI, respectively was noticed from the 1.5 mg/L BA which was statistically similar with 2.0 mg/L BA but different from the rest of the treatments. Whereas, no sprouting of shoot was observed at 3 WAI and 5 WAI in control treatment. The minimum length of shoot 1.31 cm at 8 WAI respectively was noticed in control treatment (Figure 3). Bhat *et al*. (1995), reported that BA was effective for the multiplication of *in vitro* raised plants of *Piper longum*, *Piper betel* and *Piper nigrum.*



*I= Standard error bar

Figure 3. Effect of BA on length of shoot in Black pepper

4.1.5 Number of leaves

There was a significant influence of different concentrations of BA on the number of leaves per plantlet. The treatment BA 2.0 mg/L gave the maximum number of

leaves (1.8, 2.0 and 3.2) at 3 WAI, 5 WAI and 8 WAI respectively (Plate 3). Then leaves number (1.0, 1.2 and 1.8), (1.0, 1.2 and 2.2) and (1.2, 1.2 and 2.2) was found in BA 0.5 mg/L, 1.0 mg/L and 1.5 mg/L at 3 WAI, 5 WAI and 8 WAI respectively (Table 2). As no shoot was produced in controlled treatment, so no leaf was also produced in controlled treatment at 3 WAI and 5 WAI. Whereas, the controlled treatment showed the lowest number of leaves 1.2 at 8 WAI (Table 2). Legesse et al. (2017) reported the maximum number of leaves (3.16) on BA concentration on 3mg/L of BA, but the minimum number of leaves (1.62) was recorded on controlled treatment in Black pepper. Increasing the amount of cytokinins like BA up to 5.0 mg/L gave as the maximum number of leaves per shoot in *Piper nigrum* as reported by Soniya and Das, (2002) which is partially similar with the result.

Table 2. Effect of different concentration of BA on number of leaf at different WAI

BA mg/L	**Number of leaf**		
	3 WAI	**5 WAI**	**8 WAI**
0	0 b	0 b	1.2 c
0.5	1.0 b	1.2 b	1.8 b
1.0	1.0 b	1.2b	2.2 b
1.5	1.2 b	1.2 b	2.2 b
2.0	1.8 a	2.0 a	3.2 a
CV %	28.28	30.93	21.09
LSD (0.05)	0.37	0.46	0.59

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. Number of leaves at 8 WAI in the treatment of 2.0 mg/L BA

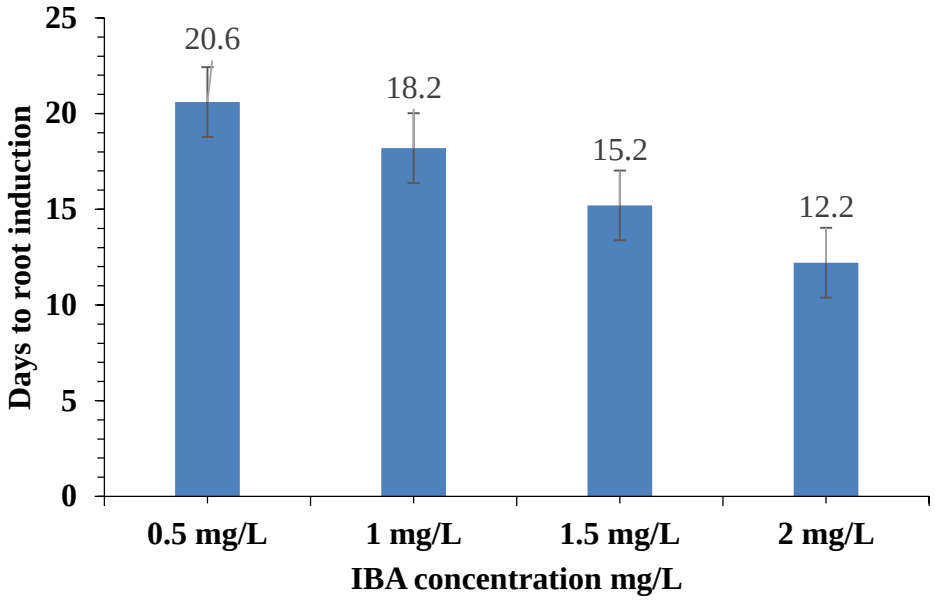
4.2 Sub-experiment 2.

Effect of IBA on root induction potentiality in Black pepper

The results of the effect of different concentrations of IBA have been presented under following headings with Figure (4-6), Table 3 and Plate 4.

4.2.1 Days to root induction

Significant variations were observed among different concentrations of IBA on days to root induction. Root induction was not recorded in controlled treatment. On the other hand, minimum 12.2 days were required in 2.0 mg/L IBA (Figure 4). The treatment 1.0 mg/L and 1.5 mg/L took 18.2 days and 15.2 days, respectively for root induction. Maximum 20.6 days were required in 0.5 mg/L IBA (Figure 4). Sanatombi and Sharma, (2007) reported that the lowest number of days was required for root initiation in 0.5 mg/L IBA of *Capsicum annuum* L.

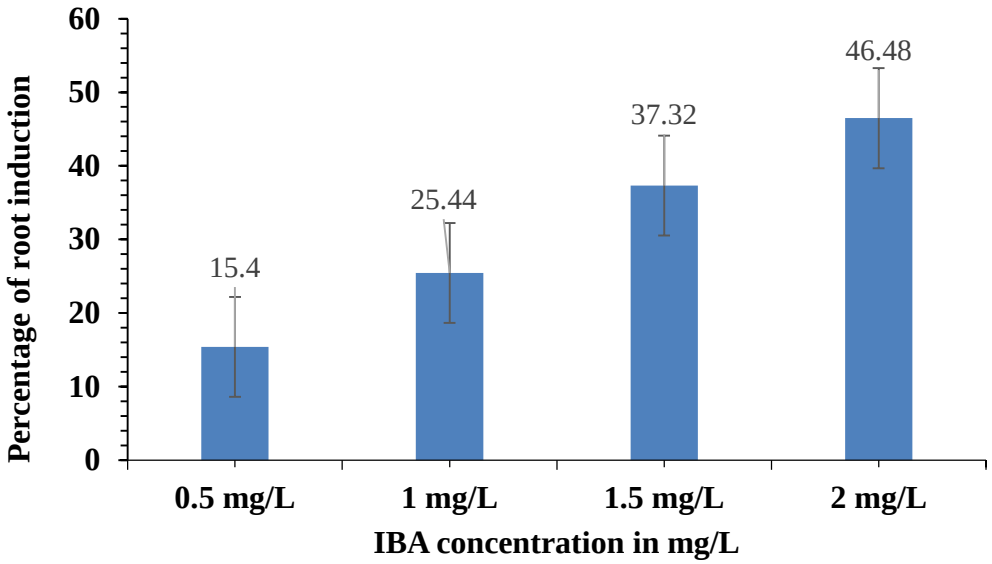


*I= Standard error bar

Figure 4. Effect of IBA on days to root induction in Black pepper

4.2.2 Percentage of root induction

The treatment 2.0 mg/L IBA had produced the optimum percentage of root induction (46.48%), while the lowest percentage (15.4%) of root induction was produced in 0.5 mg/L (Figure 5). Khan *et al*. (2017), observed that excellent rooting (80%) was obtained when shoots were cultured on half strength of MS medium with 1.5 mg/L IBA in Black pepper. Variation may be due to the age, nature and the physiological state of the explant and seasonal variation play a crucial role in the establishment of cultures and subsequent plant regeneration (Bajaj *et al*., 1991)



*I= Standard error bar

Figure 5. Effect of IBA on percentage of root induction in Black pepper

4.2.3 Number of root per shoot

There was significant influence of different concentrations of IBA on the number of roots per shoot. The treatment 2.0 mg/L gave the highest number of root (2.0, 3.0 and 4.0) at 3 WAI, 5 WAI and 8 WAI (Plate 4). Whereas (1.2, 1.8 and 2.2), (1.0, 2.0 and 2.0) and (1.6, 2.2 and 3.0) root were observed in 0.5mg/L, 1.0 mg/L and 1.5 mg/L at 3 WAI, 5 WAI and 8 WAI, respectively (Table 3). Chandrasekara *etal.* (2011), reported highest number of roots on 1.0 mg/L IBA in Black pepper in MS medium. Which is quite similar to the result. Furthermore, Garcia-Saucedo *et al*. (2005) indicated that IBA interacted significantly with the culture mediumand the materials, having a strong influence for plantlet rooting.

Table 3. Effect of different concentration of IBA on number of root at different WAI

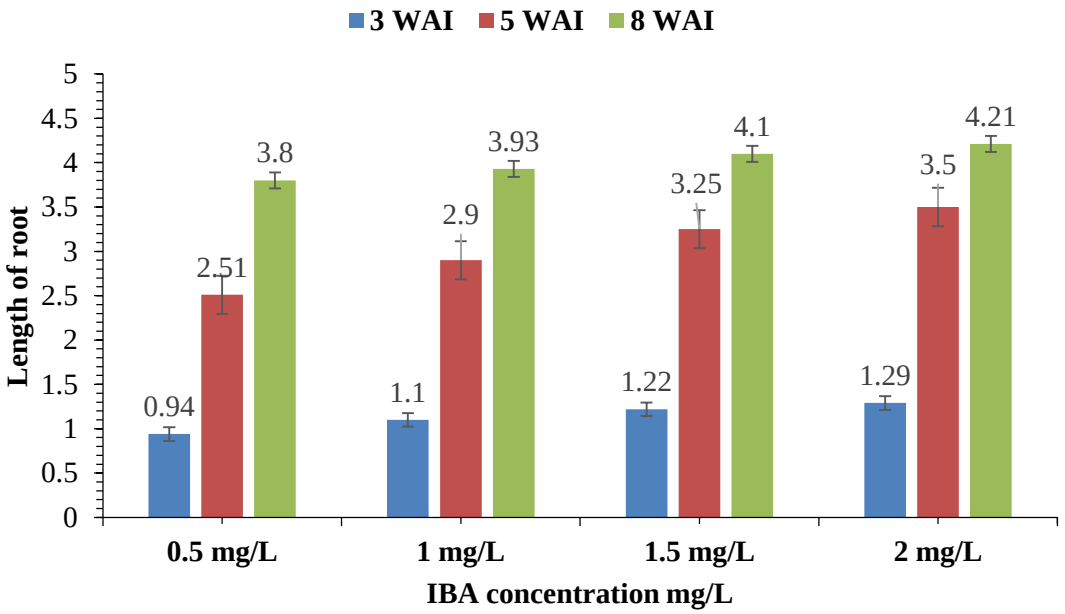
IBA mg/L	No of roots per shoot		
	3WAI	5WAI	8WAI
0.5	1.2 bc	1.8 bc	2.2 c
1.0	1.0 c	2.0 bc	2.0 c
1.5	1.6 ab	2.2 b	3.0 b
2.0	2.0 a	3.0 a	4.0 a
CV %	24.38	21.08	7.99
LSD (0.05)	0.47	0.64	0.30

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. Root development in the treatment of 2.0 mg/L IBA

4.2.4 Length of root

There was an influence of different concentrations of IBA on the length of root at 5% level of significance. The highest length of root (1.29 cm, 3.50 cm and 4.21 cm) at 3 WAI, 5 WAI and 8 WAI, respectively (Figure 6.) was noticed from 2.0 mg/L IBA. Then 0.5 mg/L, 1.0 mg/L and 1.5 mg/L of IBA treatment showed (0.94 cm, 2.51 cm and 3.80 cm), (1.10 cm, 2.90 cm and 3.93 cm) and (1.22 cm, 3.25 cm and 4.10 cm) length of root at 3 WAI, 5 WAI and 8 WAI respectively. On the other hand, media without no hormone produced no root. In Beetle Vine, IBA at 3 mg/L shows the best result as rooting hormone (Elahi et al., 2017). It isquite similar to this result.



*I= Standard error bar

Figure 6. Effect of IBA on length of root in Black pepper

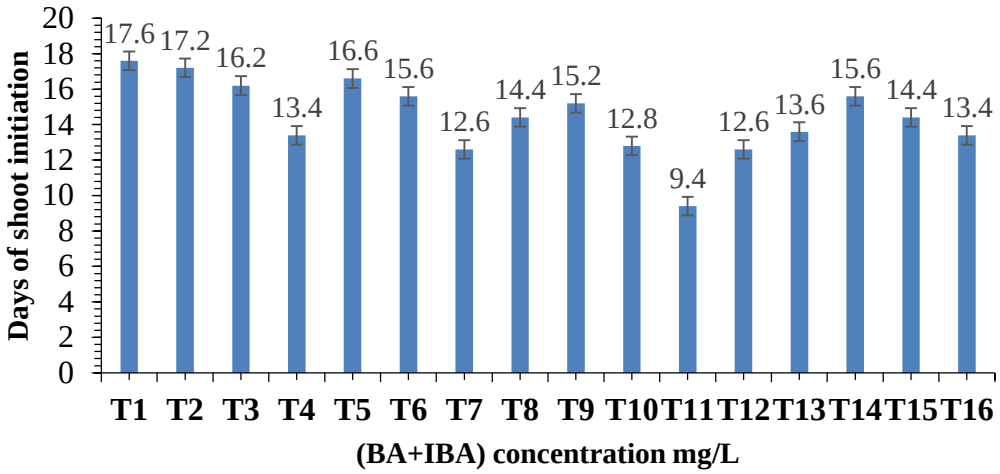
4.3 Sub-experiment 3.

Effect of BA and IBA on *in vitro* shoot and root induction potentiality in Black pepper

The results of the combined effect of different concentrations of BA + IBA have been presented under following headings with Figure (7-11), Table (4-7) and Plate (5- 9).

4.3.1 Days to shoot initiation

Significant variations were required among the combined effect of different concentrations of BA and IBA on days to shoot induction. The minimum duration 9.4 days was obtained in 2.0 mg/L BA+ 2.0 mg/L IBA than rest of the treatments. The maximum duration 17.6 days was obtained in BA 1.0 mg/L+IBA 1.0 mg/L (Figure 7). As a very few number of shoots and no root were not produced in controlled treatment in Sub-experiment 1. and Sub- experiment 2. Controlled treatment was not practiced in Sub-experiment 3. Khan *et al.* (2017), reported that in BAP 1.0 mg/L and IAA 1.0 mg/L supplemented medium 10 - 20 days were required for shoot initiation in Black pepper.



*I= Standard error bar

Figure 7. Combined effect of BA and IBA on days to shoot initiation in Black pepper

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 maximum percentage (61.28%) of shoot induction was noticed in treatment BA 2.0 mg/L + IBA 2.0 mg/L which was the best than any other treatment and minimum percentage (31.68%) was noticed in BA 1.5 mg/L+ BA 1.5 mg/L (Table 4). Khan *et al.* (2017), reported that 80% shooting response were recorded in MS + 1.0 mg/L and BAP + 1.0 mg/L in Black pepper. Which partially similar with the result. Variation may be due to growth regulators in the culture media, genetic, physiological and morphological change *in vitro* (Chaturvedi *et al.* 2007).

Table 4. Effect of different concentration of BA and IBA on days to shoot initiation and percentage of shoot induction

SL No.	Treatment BA+IBA (mg/L)	Days to shoot initiation	Percentage of shoot induction
1.	1.0+1.0	17.6 a	40.40
2.	1.0+1.5	17.2 ab	33.16
3.	1.0+2.0	16.2 cd	41.12
4.	1.0+2.5	13.4 gh	47.52
5.	1.5+1.0	16.6 bc	32.70
6.	1.5+1.5	15.6 de	31.68
7.	1.5+2.0	12.6 i	48.38
8.	1.5+2.5	14.4 f	42.86
9.	2.0+1.0	15.2 e	54.58
10.	2.0+1.5	12.8 hi	57.94
11.	2.0+2.0	9.4 j	61.28
12.	2.0+2.5	12.6 i	45.14
13.	2.5+1.0	13.6 g	42.20
14.	2.5+1.5	15.6 de	43.36
15.	2.5+2.0	14.4 f	57.54
16.	2.5+2.5	13.4 gh	56.08
	CV %	3.84	-
	LSD (0.05)	0.6992	-

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 plantlet

Different concentrations of BA and IBA showed significant variations on the number of shoot. The highest number of shoot (1.8, 2.4 and 3.2) was noticed from the 2.0 mg/L BA + 2.0 mg/L IBA (Plate 5) and second highest number

(1.4, 1.8 and 2.6) at 3 WAI, 5 WAI and 8 WAI respectively, were observed in 2.5 mg/L BA + 2.5 mg/L IBA. Whereas the lowest average number of shoot (1.0, 1.2 and 1.4) at 3 WAI, 5 WAI and 8 WAI respectively were noticed in 1.0 mg/L BA +1.5 mg/L IBA (Table 5). Monney *et al.* (2016), reported that highest numberof shoot 1.28 was found in 3.0 mg/L BA + 0.1 mg/L IBA in *Cryptolepis sanguinolenta*. Which is contradicts with the result.

Table 5. Effect of BA and IBA on number of shoot at different WAI

SL No.	BA+IBA (mg/L)	Number of shoot		
		3 WAI	5 WAI	8 WAI
1.	1.0+1.0	1.2 b	1.4 cd	1.6 ef
2	1.0+1.5	1.0 b	1.2 d	1.4 f
3.	1.0+2.0	1.2 b	1.4 cd	2.0 cde
4.	1.0+2.5	1.4 ab	1.4 cd	1.6 ef
5.	1.5+1.0	1.4 ab	1.8 bc	2.0 cde
6.	1.5+1.5	1.2 b	1.8 bc	1.8 def
7.	1.5+2.0	1.4 ab	2.0 ab	2.0 cde
8.	1.5+2.5	1.2 b	2.0 ab	2.0 cde
9.	2.0+1.0	1.4 ab	2.0 ab	2.4 bc
10	2.0+1.5	1.4 ab	1.6 bcd	2.0 cde
11.	2.0+2.0	1.8 a	2.4 a	3.2 a
12.	2.0+2.5	1.2 b	1.6 bcd	2.0 cde
13.	2.5+1.0	1.2 b	1.6 bcd	2.2 bcd
14.	2.5+1.5	1.2 b	1.4 cd	2.2 bcd
15.	2.5+2.0	1.2 b	1.6 bcd	2.2 bcd
16.	2.5+2.5	1.4 b	1.8 bc	2.6 b
	CV %	36.49	27.72	19.05
	LSD (0.05)	0.60	0.59	0.50

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.

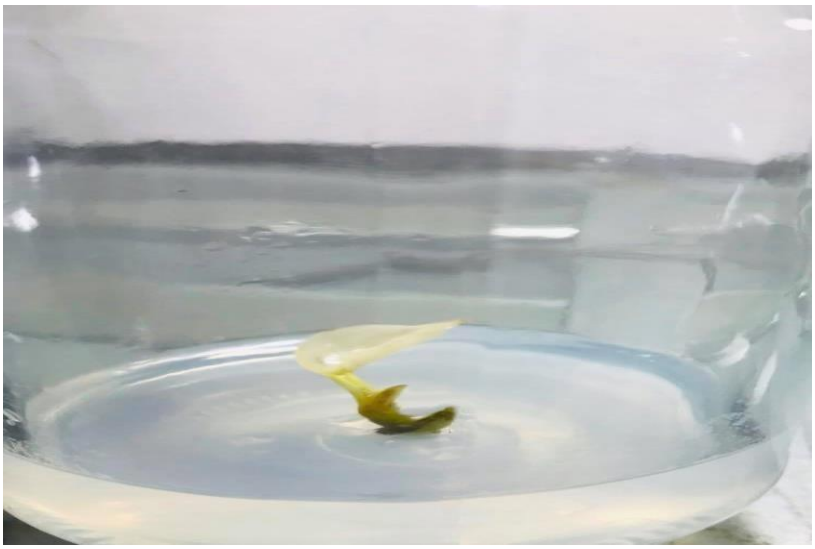


Plate 5. Number of shoot at 8 WAI in the treatment of BA 2.0 mg/L + IBA 2.0 mg/L

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 (1.91 cm, 3.23 cm and 5.10 cm) at 3 WAI, 5 WAI and 8 WAI, respectively was noticed from the BA 2.0 mg/L + IBA 2.0 mg/L (Plate 6) followed by 2.0 mg/L BA + 2.5mg/L IBA (1.85 cm, 2.33 cm and 2.86 cm) and 2.0 mg/L BA+ 1.5 mg/L IBA (1.41 cm, 2.33 cm and 2.85 cm) at 3 WAI, 5 WAI and 8 WAI, respectively. Whereas the minimum length of shoot (1.36 cm, 2.16 cm and 2.40 cm) at 3 WAI, 5 WAI and 8 WAI respectively were noticed in 1.0 mg/L BA + IBA 1.0 mg/L IBA (Table 6).

Table 6. Effect of different concentration BA and IBA on length of shoot

SL. No.	Treatment BA+IBA	Length of shoot		
		3 WAI	5 WAI	8 WAI
1.	1.0+1.0	1.36 jk	2.16 d	2.40 e
2.	1.0+1.5	1.35 l	2.33 c	2.73 c
3.	1.0+2.0	1.34 l	2.33 c	2.75 c
4.	1.0+2.5	1.37 j	2.34 c	2.72 c
5.	1.5+1.0	1.71 e	2.53 b	2.83 b
6.	1.5+1.5	1.74 d	2.53 b	2.83 b
7.	1.5+2.0	1.76 c	2.54 b	2.83 b
8.	1.5+2.5	1.75 cd	2.52 b	2.82 b
9.	2.0+1.0	1.41 i	2.33 c	2.85 b
10.	2.0+1.5	1.41 i	2.34 c	2.85 b
11.	2.0+2.0	1.91 a	3.23 a	5.10 a
12.	2.0+2.5	1.85 b	2.33 c	2.86 b
13.	2.5+1.0	1.51 g	2.14 de	2.50 d
14.	2.5+1.5	1.61f	2.16 d	2.53 d
15.	2.5+2.0	1.44 h	2.15 de	2.53 d
16.	2.5+2.5	1.35 kl	2.12 e	2.54 d
	CV %	0.66	1.35	3.02
	LSD (0.05)	0.01	0.04	0.10

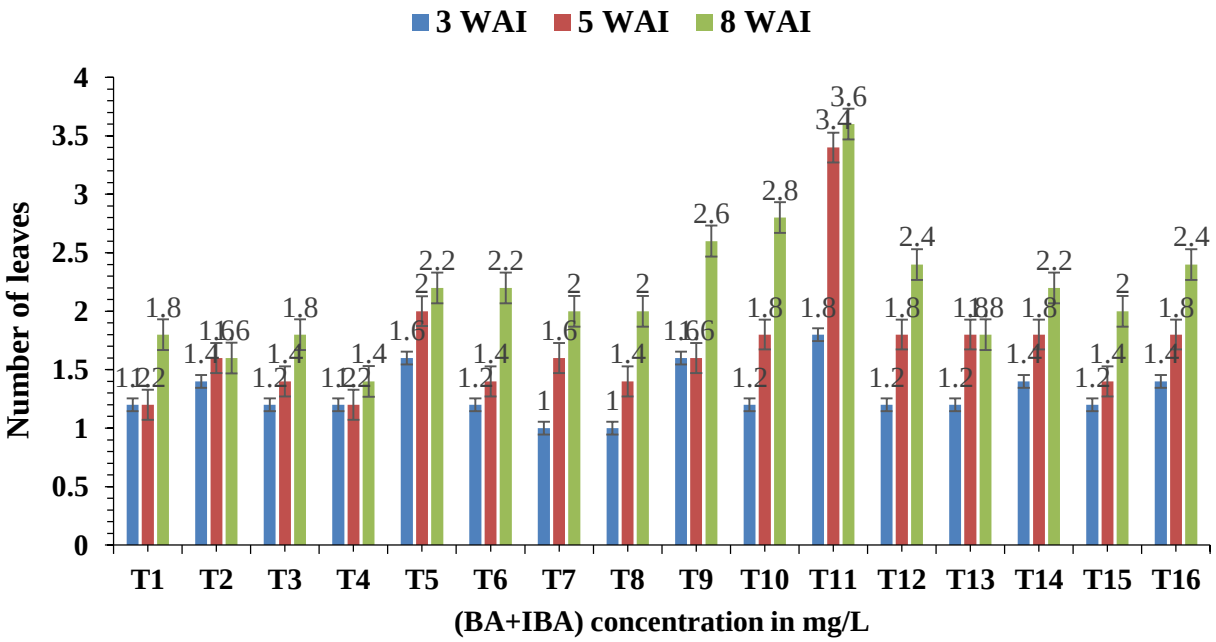
Figures in a column followed by different letter(s) differs significantly whereas figures having common letter(s) do not differ significantly from each other as adjusted by DMRT. CV= Coefficient of variation, LSD (0.05) = Least significant difference.

Plate 6. Length of shoot at 8 WAI in the treatment of BA 2.0 mg/l + IBA 2.0 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 2.0 mg/L BA + 2.0 mg/L IBA gave the highest number of leaves (1.8, 3.4 and 3.6) at 3 WAI, 5 WAI and 8 WAI respectively (Plate 7) whereas the lowest number of leaves (1.2, 1.2 and 1.4) at 3 WAI, 5 WAI and 8 WAI respectively was found in BA 1.0 mg/L+ IBA 2.5 mg/L (Figure 8).

Plate 7. Number of leaves at 8 WAI in the treatment of 2.0 mg/L BA + 2.0 mg/L IBA

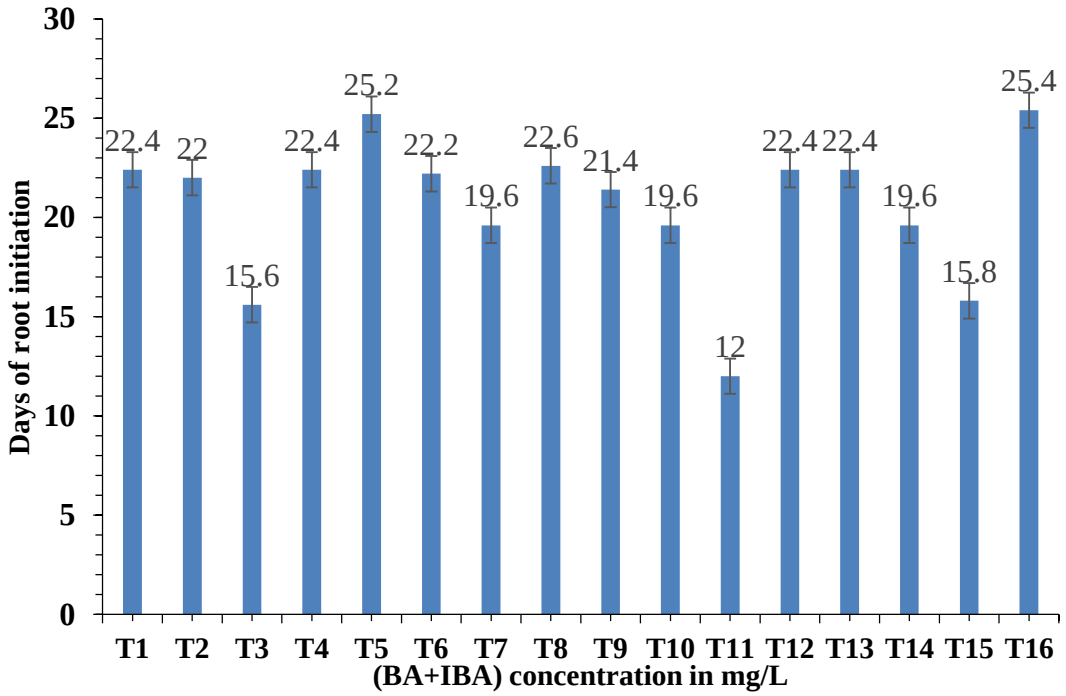


*I= Standard error bar

Figure 8. Combined effect of BA and IBA on number of leaves in Black pepper

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 25.4 days to root induction was recorded in 2.5 mg/L BA+ 2.5 mg/L IBA and minimum 12.0 days was required in 2.0 mg/L BA + 2.0 mg/L IBA (Figure 9). Khalafallh *et al*. (2007) reported that the minimum 5.6 days was observed to induced root in *Opuntia fiscus* species with BA (2.4 mg/L) + IBA (1.8 mg/L). The findings of this studyis quite similar to their results.



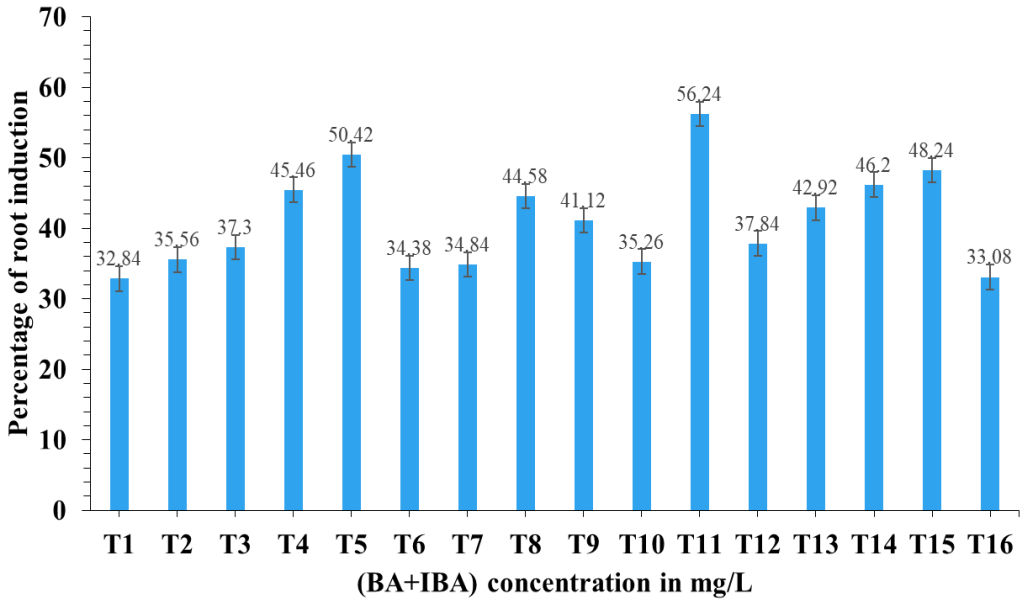
*I= Standard error bar

Figure 9. Combined effect of BA and IBA on days to root initiation in Black pepper

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 (56.24%) of root induction was recorded with 2.0 mg/L BA + 2.0 mg/L IBA, whereas the lowest percentage (32.84%) of root induction was recorded in 1.0 mg/L BA +

1.0 mg/L IBA (Figure 10). Myeong *et al*. (2004) noticed that the maximum percentage (92%) of root induction was observed in BA (2.5 mg/L) + IBA (1.8 mg/L) in Cacti.

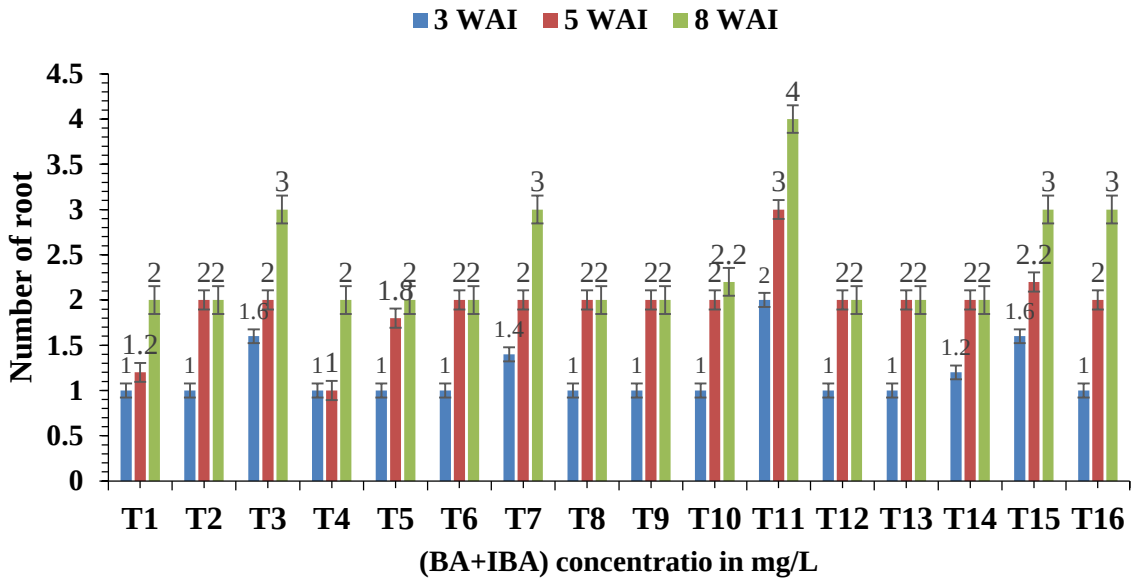


*I= Standard error bar

Figure 10. Combined effect of BA and IBA on percentage of root induction in Black pepper

4.3.8 Number of root per shoot

There was a significant influence of different concentrations of BA and IBA on the number of root per shoot. The treatment 2.0 mg/L BA + 2.0 mg/L IBA gave the highest number of root (2.0, 3.0 and 4.0) (Plate 8) and second best result (1.6, 2.2 and 3.0) was noticed from 2.5 mg/L BA+ 2.0 mg/L IBA at 3 WAI, 5 WAI and 8 WAI whereas the lowest number of root (1.0, 1.0 and 2.0) at 3 WAI,5 WAI and 8 WAI was found in 1.0 mg/L BA +2.5 mg/L IBA (Figure 11).



*I= Standard error bar

Figure 11. Combined effect of BA and IBA on number of root in Black pepper

Plate 8. Number of root at 5 WAI in the treatment of 2.0 mg/L BA + 2.0 mg/L IBA

4.3.9 Length of root (cm)

There was a significant variation at 3 WAI, 5 WAI and 8 WAI among different concentration of BA and IBA on length of root. The highest length of root (1.25 cm, 3.24 cm and 4.29 cm) at 3 WAI, 5 WAI and 8 WAI, respectively was found in 2.0 mg/L BA + 2.0 mg/L IBA (Plate 9). The second highest result was observed in MS media with 1.5 mg/L BA + 2.0 mg/L IBA (0.85 cm, 2.11 cm and 3.41 cm) at 3 WAI, 5 WAI and 8 WAI, respectively (Table 7). The lowest number of root (0.47 cm, 1.24 cm and 2.23 cm) at 3 WAI, 5 WAI and 8 WAI, respectively was observed in 1.0 BA mg/L+2.5 mg/L IBA (Table 7).

Table 7. Effect of BA and IBA on length of root at different WAI

SL. No.	Treatment BA+IBA	Length of root (cm)		
		3 WAI	5 WAI	8 WAI
1.	1.0+1.0	0.60 j	1.20 k	2.53 h
2.	1.0+1.5	0.76 f	1.64 e	2.84 f
3.	1.0+2.0	0.87 b	2.09 c	3.35 c
4.	1.0+2.5	0.47 m	1.24 h	2.23 k
5.	1.5+1.0	0.65 h	1.28 g	2.54 h
6.	1.5+1.5	0.80 e	1.74 d	2.94 e
7.	1.5+2.0	0.85 c	2.11 b	3.41 b
8.	1.5+2.5	0.83 d	1.34 f	3.14 d
9.	2.0+1.0	0.63 I	1.23 I	2.54 h
10.	2.0+1.5	0.74 g	1.64 e	2.85 f
11.	2.0+2.0	1.25 a	3.24 a	4.29 a
12.	2.0+2.5	0.84 c	1.34 f	3.15 d
13.	2.5+1.0	0.50 l	1.10 l	2.42 I
14.	2.5+1.5	0.52 k	1.22 j	2.55 h
15.	2.5+2.0	0.65 h	1.28 g	2.77 g
16.	2.5+2.5	0.45 n	1.10 l	2.40 j
	CV %	0.98	0.43	0.44
	LSD (0.05)	8.82	8.42	0.02

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.

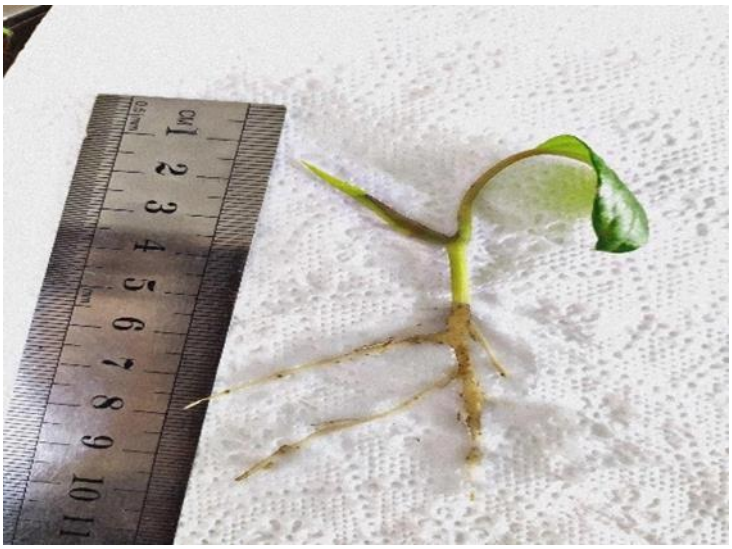


Plate 9. Length of root at 8 WAI in the treatment of 2.0 mg/L BA+ 2.0mg/L IBA

Sub-experiment 4.

Acclimatization and establishment of plantlets on soil

After considerable shoots and roots were developed at 8 weeks of culture, the plantlets were removed from vial carefully without any root damage.

Table 8. Survival rate of *in vitro* regenerated plantlet of Black pepper

Acclimatization	**No. of planlet transferred**	**No. of Plantlet survive**	**Survival rate (%)**
In shade house with controlled environment	15.0	7.0	46.67
In natural condition	7.0	4.0	57.14

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 relative humidity favourable condition for plant establishment. In the meantime, the plantlets transferred to vermiculite pot filled with sterilized soil: cow dung (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 was 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 tothe field conditions. At first 15 plants were transplanted and 7 were survived in shade condition (46.67%). Finally, in normal atmospheric condition 7 plants were transplanted among them 4 survived and survival rate was 57.14%. Anand *et al*. (2000) reported that in natural condition 75% plantlets survived. Padhan (2015) observed 90% plantlets survival in soil in shade house.

Plate 10. Acclimatization of plantlets in the shade condition

Plate 11. Establishment of plantlet in natural condition

CHAPTER V

SUMMARY AND CONCLUSION

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 January to September 2020.The nodal segments of Black pepper were used as experimental materials in the present investigation. BA (0.5. 1.0, 1.5 and 2.0 mg/L) and IBA (0.5. 1.0, 1.5 and 2.0 mg/L) alone or in combination of BA (1.0, 1.5. 2.0 and 2.5mg/L) + IBA (1.0, 1.5. 2.0 and 2.5 mg/L) were used as treatment. The experiments were arranged in Completely Randomized Design (CRD) with five replications. The major findings were given below.

The treatment 1.5 mg/L BA gave the highest number of shoots (1.8, 2.6 and 3.0) at 3 WAI, 5WAI and 8 WAI, respectively whereas shoot regeneration was not observed in hormone free media at 3 WAI and 5 WAI but very incipient shoot found at 8 WAI. Again the highest percentage (52.32%) of shoot induction was noticed in treatment 1.5 mg/L BA and the lowest percentage (9.34%) was induced in the media which was hormone free. The highest number of leaves (1.8, 2.0 and 3.2) at 3 WAI, 5 WAI and 8 WAI respectively were observed from the 2.0 mg/L BA and no leaf was not observed at 3 WAI and 5 WAI in controlled treatment the lowest number of leaves 1.2 at 8 WAI were observed with controlledtreatment.

The treatment IBA 2.0 mg/L had produced the maximum percentage of root (46.48%) required minimum 12.2 days. This treatment also gave the highest number of root (2.0, 3.0 and 4.0) at 3 WAI, 5 WAI and 8 WAI respectively. The

maximum length of root (1.29 cm, 3.50 cm and 4.21 cm) at 3 WAI, 5 WAI and 8 WAI respectively was found in 2.0 mg/L IBA.

In combined effect, the maximum percentage (61.28%) of shoot induction was observed in 9.4 days with treatment BA (2.0 mg/L) + IBA (2.0 mg/L). The minimum percentage (31.68%) was observed in BA (1.5 mg/L) + IBA (1.5 mg/L) and the maximum 17.6 days in BA (1.0 mg/L) + IBA (1.0 mg/L) treatment. The treatment BA (2.0 mg/L) + IBA (2.0 mg/L) gave the maximum number of shoots (1.8, 2.4 and 3.2) at 3 WAI, 5 WAI and 8 WAI respectively whereas the minimum number of shoots (1.0, 1.2 and 1.4) at 3 WAI, 5 WAI and 8 WAI respectively and were observed in BA (1.0 mg/L) + IBA (1.5 mg/L). The treatment BA (2.0 mg/L) + IBA (2.0 mg/L) gave the maximum length of shoot (1.91 cm, 3.23 cm and 5.10 cm) at 3 WAI, 5 WAI and 8 WAI respectively whereas the minimum length of shoots (1.36 cm, 2.16 cm and 2.40 cm) at 3 WAI, 5 WAI and 8 WAI respectively were observed in BA (1.0 mg/L) + IBA (1.0 mg/L) respectively. Then the maximum number of leaves (1.8, 3.4 and 3.6) at 3 WAI, 5 WAI and 8 WAI respectively were observed from the treatment of 2 mg/L BA + 2 mg/L IBA. Again the minimum number of leaves (1.2, 1.2 and 1.4) at 3 WAI, 5 WAI and 8 WAI respectively were observed from the BA (1.0 mg/L) + IBA (2.5 mg/L) respectively.

Again in combined effect, the maximum percentage (56.24%) of root was recorded in 12 days with the treatment of BA (2.0 mg/L) + IBA (2.0 mg/L). The minimum percentage (32.84%) was observed in BA (1.0 mg/L) + IBA (1.0 mg/L) and maximum 25.4 days in BA (2.5 mg/L) + IBA (2.5 mg/L). The maximum number of root (2.0, 3.0 and 4.0) at 3 WAI, 5WAI and 8 WAI was observed with the treatment of BA (2.0 mg/L)+ IBA (2.0 mg/L) whereas the minimum number of root (1.0, 1.0 and 2.0) at 3 WAI, 5 WAI and WAI respectively were observed with the treatment of BA (1.0 mg/L) + IBA (2.5 mg/L). The maximum length of

roots (1.25 cm, 3.24 cm and 4.29 cm) at 3 WAI, 5 WAI and WAI respectively were observed with the treatment of BA (2.0 mg/L) + IBA (2.0 mg/L) whereas the minimum length of roots (0.47 cm, 1.24 cm and 2.23 cm) at 3 WAI, 5 WAI and 8 WAI respectively were observed BA (1.0 mg/L) + IBA (2.5 mg/L) respectively.

Regenerated plantlets showed survival during 46.67% in shade house and 57.14% in open atmosphere. In conclusion, regenerated plants were found to be morphologically similar to the mother plant. A convenient *in vitro* regeneration protocol had been established. Findings of the present study showed that *in vitro* regeneration is an effective method for the micropropagation of Blackpepper. Thus the protocol of *in vitro* rapid regeneration of Black pepper has been established which may contribute in large seedling production throughout the year.

RECOMMENDATIONS

The following recommendations could be addressed based on the present experiment,

i. Other explants such as meristem and root tip can be experimented for the proliferation of Black pepper.

ii. Further study can be done with different concentrations and combinations of auxin and cytokinine group of hormones for the micropropagation of Black pepper.

iii. To understand the influence of genotype, more research should be carried out with different types of genotypes of Black pepper.

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APPENDICES

Appendix 1. Composition of Duchefa Biochemic MS (Murashige and Skoog, 1962) medium including vitamins

Components	Concentrations (mg/L)	Concentrations
Micro Elements	**mg/L**	**Mm**
$CoCl_2.6H_2O$	0.025	0.11
$CuSO_4.5H_2O$	0.025	0.10
Fe Na EDTA	36.70	100.0
H_3BO_3	6.20	100.27
KI	0.83	5.00
$MnSO_4.H_2O$	16.90	100.0
$Na_2MoO_4.2H_2O$	0.25	1.03
$ZnSO_4.7H_2O$	8.60	29.91
Macro Elements	**mg/L**	**Mm**
$CaCl_2$	332.02	2.99
KH_2PO_4	170.00	1.25
KNO_3	1900.00	18.79
$MgSO_4$	180.54	1.50
NH_4NO_3	1650.00	20.61
Vitamins	**mg/L**	**Mm**
Glysine	2.00	26.64
Myo-Inisitol	100.0	554.94
Nicotinic Acid	0.50	4.06
Pyridoxine HCl	0.50	2.43
Thiamine	0.10	0.30

Total concentration of Micro and Macro elements including vitamins: 4405.19 mg/L

Manufacturing Company: Duchefa Biochem

Appendix II. Analysis of variances on days to shoot induction with BA

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-Value	Probability
Trt	4	228.560	57.1400	219.77	0.0000
Error	20	5.200	0.2600		
Total	24	233.760			
CV%	3.48				
LSD value	0.6727				

Appendix III. Analysis of variance (ANOVA) on number of shoot with BA in 3 WAI

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-Value	Probability
Trt	4	8.40000	2.10000	26.25	0.0000
Error	20	1.60000	0.08000		
Total	24	10.00000			
CV%	28.28				
LSD Value	0.3731				

Appendix IV. Analysis of variance (ANOVA) on number of shoot with BA in 5 WAI

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-Value	Probability
Trt	4	19.0400	4.76000	29.75	0.0000
Error	20	3.2000	0.16000		
Total	24	22.2400			
CV%	27.03				
LSD Value	0.5277				

Appendix V. Analysis of variance (ANOVA) on number of shoot with BA in 8 WAI

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-Value	Probability
Trt	4	10	2.5	25	0.0000
Error	20	2	0.1		
Total	24	12			
CV%	15.81				
LSD Value	0.4172				

Appendix VI. Analysis of variance (ANOVA) on length of shoot with BA in 3 WAI

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-Value	Probability
Trt	4	14.2809	3.57023	93953.53	0.0000
Error	20	0.0008	0.00004		
Total	24	14.2817			
CV%	0.41				
LSD Value	8.133				

Appendix VII. Analysis of variance (ANOVA) on length of shoot with BA in 5 WAI

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-Value	Probability
Trt	4	19.8278	4.95695	9388.17	0.0000
Error	20	0.0106	0.00053		
Total	24	19.8384			
CV%	1.30				
LSD Value	0.0303				

Appendix VIII. Analysis of variance (ANOVA) on the length of shoot with BA 8 WAI

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-Value	Probability
Trt	4	8.36426	2.09106	59085.11	0.0000
Error	20	0.00072	0.00004		
Total	24	8.36498			
CV%	0.24				
LSD Value	7.916				

Appendix IX. Analysis of variance (ANOVA) on the number of leaf with BA in 3 WAI

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-Value	Probability
Trt	4	8.40000	2.10000	26.25	0.0000
Error	20	1.60000	0.08000		
Total	24	10.00000			
CV%	28.28				
LSD Value	0.3731				

Appendix X. Analysis of variance (ANOVA) on the number of leaf with BA in 5WAI

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-Value	Probability
Trt	4	10.24000	2.56000	21.33	0.0000
Error	20	2.40000	0.12000		
Total	24	12.64000			
CV%	30.93				
LSD Value	0.4570				

Appendix XI. Analysis of variance (ANOVA) on the number of leaf with BA in 8 WAI

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-Value	Probability
Trt	4	10.6400	2.66000	13.30	0.0000
Error	20	4.0000	0.20000		
Total	24	14.6400			
CV%	21.09				
LSD Value	0.5900				

Appendix XII. Analysis of variance (ANOVA) on days of root initiation with IBA

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-Value	Probability
Trt	3	199.350	66.4500	295.33	0.0000
Error	16	3.600	0.2250		
Total	19	202.950			
CV%	2.87				
LSD Value	0.6360				

Appendix XIII. Analysis of variance (ANOVA) on the number of root with IBA in 3 WAI

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-Value	Probability
Trt	3	2.95000	0.98333	7.870	0.0019
Error	16	2.00000	0.12500		
Total	19	4.95000			
CV%	24.38				
LSD Value	0.4740				

Appendix XIV. Analysis of variance (ANOVA) on the number of root with IBA in 5 WAI

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-Value	Probability
Trt	3	4.1500	1.38333	6.15	0.0055
Error	16	3.6000	0.22500		
Total	19	7.7500			
CV%	21.08				
LSD Value	0.6360				

Appendix XV. Analysis of variance (ANOVA) on the number of root with IBA in 8WAI

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-Value	Probability
Trt	3	12.400	4.12000	82.67	0.0000
Error	16	0.8000	0.05000		
Total	19	13.200			
CV%	7.99				
LSD Value	0.2998				

Appendix XVI. Analysis of variance (ANOVA) on the length of root with IBA in 3 WAI

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-Value	Probability
Trt	3	0.33846	0.11282	2256.37	0.0000
Error	16	0.00080	0.00005		
Total	19	0.33926			
CV%	0.62				
LSD Value	9.481				

Appendix XVII. Analysis of variance (ANOVA) on the length of root with IBA in 5 WAI

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-Value	Probability
Trt	3	2.81804	0.93935	31311.56	0.0000
Error	16	0.00048	0.00003		
Total	19	2.81852			
CV%	0.18				
LSD Value	7.344				

Appendix XVIII. Analysis of variance (ANOVA) on the length of root with IBA in 8 WAI

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-Value	Probability
Trt	3	0.50978	0.16993	6797.07	0.0000
Error	16	0.00040	0.00003		
Total	19	0.51018			
CV%	0.12				
LSD Value	6.704				

Appendix XIX. Analysis of variance (ANOVA) on the days of shoot initiation with BA +IBA

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-Value	Probability
Trt	15	331.788	22.1192	72.23	0.0000
Error	64	19.600	0.3062		
Total	79	351.388			
CV%	3.84				
LSD Value	0.6992				

Appendix XX. Analysis of variance (ANOVA) on number of shoot with BA +IBA in 3 WAI

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-Value	Probability
Trt	15	2.4000	0.16000	0.71	0.7642
Error	64	14.4000	0.22500		
Total	79	16.8000			
CV%	36.49				
LSD Value	0.5993				

Appendix XXI. Analysis of variance (ANOVA) on number of shoot with BA +IBA in 5 WAI

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-Value	Probability
Trt	16	7.1875	0.58824	2.19	0.0158
Error	64	14.0000	0.21875		
Total	79	21.1875			
CV%	27.72				
LSD Value	0.5909				

Appendix XXII. Analysis of variance (ANOVA) on number of shoot with BA +IBA in 8 WAI

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-Value	Probability
Trt	15	13.5500	0.90333	5.78	0.0000
Error	64	10.0000	0.15625		
Total	79	23.5500			
CV%	19.05				
LSD Value	0.4994				

Appendix XXIII. Analysis of variance (ANOVA) on length of shoot with BA +IBA in 3 WAI

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-Value	Probability
Trt	15	3.02471	0.20165	1931.95	0.0000
Error	64	0.00668	0.00010		
Total	79	3.03139			
CV%	0.66				
LSD Value	0.0129				

Appendix XXIV. Analysis of variance (ANOVA) on length of shoot with BA +IBA in 5 WAI

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-Value	Probability
Trt	15	5.48777	0.36585	366.08	0.0000
Error	64	0.06396	0.00100		
Total	79	5.55173			
CV%	1.33				
LSD Value	0.0399				

Appendix XXV. Analysis of variance (ANOVA) on length of shoot with BA +IBA in 8 WAI

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-Value	Probability
Trt	15	28.7252	1.91501	845.71	0.0000
Error	64	0.1449	0.00226		
Total	79	28.8701			
CV%	1.67				
LSD Value	0.0601				

Appendix XXVI. Analysis of variance (ANOVA) on number of leaf with BA +IBA in 3 WAI

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-Value	Probability
Trt	15	3.6000	0.2400	1.16	0.3225
Error	64	13.2000	0.20625		
Total	79	16.8000			
CV%	34.93				
LSD Value	0.2872				

Appendix XXVII. Analysis of variance (ANOVA) on number of leaf with BA +IBA in 5 WAI

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-Value	Probability
Trt	15	19.6000	1.30607	5.50	0.0000
Error	64	15.2000	0.23750		
Total	79	34.8000			
CV%	28.67				
LSD Value	0.6157				

Appendix XXVIII. Analysis of variance (ANOVA) on number of leaf with BA +IBA in 8 WAI

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-Value	Probability
Trt	15	20.7500	1.38333	4.71	0.0000
Error	64	18.8000	0.29375		
Total	79	39.5500			
CV%	24.92				
LSD Value	0.6848				

Appendix XXIX. Analysis of variance (ANOVA) on number of root with BA +IBA in 3WAI

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-Value	Probability
Trt	15	7.1500	0.47667	6.93	0.0000
Error	64	4.4000	0.06875		
Total	79	11.5500			
CV%	22.32				
LSD Value	0.3313				

Appendix XXX. Analysis of variance (ANOVA) on number of root with BA +IBA in 5 WAI

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-Value	Probability
Trt	15	17.5875	1.17250	31.27	0.0000
Error	64	2.4000	0.03750		
Total	79	19.9875			
CV%	10.26				
LSD Value	0.2447				

Appendix XXXI. Analysis of variance (ANOVA) on number of root with BA +IBA in 8 WAI

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-Value	Probability
Trt	15	27.600	1.84000	73.60	0.0000
Error	64	1.6000	0.02500		
Total	79	29.200			
CV%	6.59				
LSD Value	0.1998				

Appendix XXXII. Analysis of variance (ANOVA) on days of root initiation with BA +IBA

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-Value	Probability
trt	15	956.287	63.7525	231.83	0.0000
Error	64	17.600	0.2750		
Total	79	973.887			
CV%	2.54				
LSD Value	0.6626				

Appendix XXXIII. Analysis of variance (ANOVA) on length of root with BA +IBA in 3 WAI

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-Value	Probability
Trt	15	3.02362	0.20157	4134.86	0.0000
Error	64	0.00312	0.00005		
Total	79	3.02674			
CV%	0.98				
LSD Value	8.822				

Appendix XXXIV. Analysis of variance (ANOVA) on length of root with BA +IBA in 5 WAI

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-Value	Probability
Trt	15	22.8656	1.52438	34352.15	0.0000
Error	64	0.0028	0.00004		
Total	79	22.8685			
CV%	0.43				
LSD Value	8.417				

Appendix XXXV. Analysis of variance (ANOVA) on length of root with BA +IBA in 8 WAI

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-Value	Probability
Trt	15	19.8322	1.32214	8394.57	0.0000
Error	64	0.0101	0.00016		
Total	79	19.8422			
CV%	0.44				
LSD Value	0.0159				