IN VITRO REGENERATION OF PEPPER

(*Capsicum annuum* L.)

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BY

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CERTIFICATE

This is to certify that the thesis entitled ***"IN VITRO RAPID REGENERATION OF PEPPER (Capsicum annuum L.)"*** *submitted to the Department of Biotechnology, 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 result of a piece of bonafide research work carried out by* ***MD. MONUAR HOSSAIN,*** *Registration No.* ***12-04802*** *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 during the course of this investigation has been duly acknowledged and style of this thesis have been approved and recommended for submission.

Dated: June, 2018

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DEDICATED TO

My beloved parents

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SAU, Dhaka ***The Author***

IN VITRO REGENERATION OF PEPPER (*Capsicum annuum* L.)

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 February, 2017 to March, 2018 to evaluate the effect of different plant growth regulators (BAP and IBA) on *in vitro* plantlet regeneration of pepper. The shoot tips of pepper were used as explants which were inoculated in MS media supplemented with different concentration of BAP (1.0, 2.0, 3.0 and 4.0 mg/L) and IBA (1.0, 1.5, 2.0 and 2.5 mg/L) alone or in combination for plantlet regeneration. The experiments were arranged in CRD with three replications. The highest frequency (72%) of shoot development was observed in MS medium augmented with BAP 3.0 mg/L at minimum (18.25) days. Maximum number of shoots 3.50 mg/L was found in 35 days after shoot induction (DAI) at 3.0 mg/L BAP where the control treatment gave the lowest result. In combined effect, BAP 3.0 mg/L + IBA 2.0 mg/L showed the highest percentage (78.00%) of callus induction at in 16.00 days and the maximum size of callus (3.26 cm) was noticed at 35 days after induction. The highest number of shoot (3.76) was noticed from the same treatment at 35 DAI. The highest number of root (20.32) at 35 DAI was obtained in 2.0 mg/L IBA. The maximum length of roots (4.25 cm) was observed in 1.5 mg/L IBA

Finally, the survival rate of *in vitro* regenerated plantlets was recorded 75% in natural condition. Feasible protocol has been developed for *in vitro* rapid regeneration of pepper having potential in cell culture technology.

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LIST OF ABBREVIATED TERMS

Abbreviation		Full Word
Agril.	:	Agricultural
Biol.	:	Biological
BAP	:	6- Benzyl Amino Purine
BA	:	Benzyladenine
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 liter
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.	:	Etcetera

CHAPTER I

INTRODUCTION

Pepper (*Capsicum annuum* L.) belongs to the family Solanaceae under the genus *Capsicum*. Pepper, chili and the *Capsicum* are native to Tropical South America. Especially Brazil is thought to be the original home of peppers (Shoemaker and Teskey, 1955). The genus *Capsicum* contains about 20 species.

Peppers become extremely popular for the abundance and the kind of antioxidants they contain. Genus *Capsicum* is a rich source of phenolics (Howard *et al*., 2000 and Blanco-Ríos *et al*., 2013). Peppers are considered an important source of ascorbic acid, and it had been attributed health benefits (Howard et al., 1994; Kumar and Tata, 2009). Their attractive colors are due to the profuse synthesis of various carotenoid pigments during ripening (Hornero-Mendez *et al*., 2000). It has also several medicinal properties like anti-inflammatory, analgesic, carminative, rubefacient and also possess powerful antioxidant, anti-mutagenic, anti-tumoural, hypoglycaemic, antifungal and antimicrobial activities have also been seen (Pandey *et al*., 2012).

Vegetative propagation method has been practices for the cultivation of pepper plant. Pepper seeds characteristically have short viability and can rapidly loose germination capacity. During seedling production, plants do not always grow evenly and often become infected by pathogens, which results in lack of uniformity and insufficient quality of seedling. One of the most important limiting factors is the formation of rosette shoots or ill-defined leafy shoots which resist pepper elongation. Additionally, certain genetic variation can occur during the reproduction through seed material. The possibility of *in vitro* mass propagation of pepper can overcome the difficulties listed above, providing an unlimited number of entirely homogeneous healthy *in vitro* plantlets (Ostroshy *et al*., 2011). According to Dabauza and Pena (2001), pepper micropropagation is a

unique tool that is very convenient for maintaining the genetic homogeneity of the elite plant materials, as well as for preserving heterozygous genotypes and genetically transformed forms.

However, *in vitro* plant regeneration is essential for the rapid multiplication of disease free planting materials and is an imperative for the application of biotechnology tools to plant breeding and genetic improvement (Christou and Klee, 2004). It is also important for the conservation of genetically pure planting materials. Micropropagation is advantageous over traditional propagation as it can be used to multiply novel plants, such as those that have been genetically modified or bred through conventional plant breeding methods. It also leads to simultaneous accomplishment of rapid large-scale propagation of new genotypes (Dabauza and Pena, 2001 and Bhagowati and Changkija, 2009).

The first report on plant regeneration in the tissue culture of annual pepper (*Capsicum annuum* L.) was published over 3 decades ago by Gunay and Rao in 1978 (Phillips and Hubstenberger, 1985). The numerous experiments demonstrated the effectiveness of micropropagation in different species of pepper (*Capsicum* spp.) varies depending on the plant genotype, the type of initial explant, and the factors and conditions of *in vitro* cultures (Phillips and Hubstenberger, 1985; Christopher and Rajam, 1996 and Dabauza and Pena, 2001).

In general, it was found that both 6-benzylaminopurine (BAP) and indole acetic acid (IAA) are effective growth regulators in the first stage of organogenesis induction (Phillips and Hubstenberger, 1985; Christopher and Rajam, 1996; Gatz, 2002b; Paz and Castaneda, 2004; Sanatombi and Sharma, 2007).

Several attempts have been made on *in vitro* plant regeneration from different explants, but many of these investigations did not report satisfactory result in terms of enhanced number of shoots because

capsicum genus is recalcitrant with regards to its *in vitro* regeneration potential (Liu *et al*., 1990). Very little information is available on *in vitro* regeneration of *Capsicum annuum* L. under Bangladesh conditions. Effect of plant growth regulator on micro-propagation in *Capsicum annuum* L. need to establish. Hence , it is necessary to establish reliable *in vitro* regeneration systems for *Capsicum* sp.

The present investigation has been carried out to develop and establish a reliable protocol for *in vitro* regeneration of pepper and identify the best hormonal combination for pepper regeneration.

So, considering the above problem and prospects, the experiment was designed to fulfill the following objectives.

OBJECTIVES:

1. To assess different hormonal combination on *in vitro* regeneration of Pepper (*Capsicum annuum* L.)
2. To find out best hormonal treatment for *in vitro* regeneration.
3. To establish an efficient *in vitro* regeneration protocol of *Capsicum annuum* L.

CHAPTER II
REVIEW OF LITERATURE

2.1. Basic information on plant tissue culture:

Plant tissue culture is the latest route in the science of cell biology. Tissue culture is the process of regeneration under aseptic condition in an artificial nutrient medium. The idea of plant tissue culture originated from the cell theory and it was formulated by Schwann in 1839. Development of new organized structures i.e. organs from the old one through tissue culture is done by two ways: direct and indirect. Emergence of adventitious organs directly from explants known as direct method. Indirect is the process of regeneration new organs through callus formation. Callus is an unorganized mass of cells that develops when cells are wounded and is very useful for many *in vitro* cultures. Both BAP and IBA aid in the formation of most callus cells (Ali et al., 2007). Callus can be continuously proliferated using plant growth hormones or then directed to form organs or somatic embryos. For rapid propagation tissue culture is now applied in many sector of biology. Like many other countries in Bangladesh different government and non-government organizations are working in this area. Many plant breeders has been employing biotechnological tools for the development of pepper. 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. Explant

Fatima *et al*. (2014) reported that maximum calli (83%) of chili induced in cotyledon explant on MS medium containing 2, 4-D (1 mg/L) through *in vitro* plant regeneration. No embryogenesis could be observed in calli when sub-cultured on auxin free medium. Highest shoot regeneration

potential (53%) was observed in cotyledon leaf explant induced calli on MS medium containing BAP and Kin (3 mg/L + 1 mg/L).

2.3. Sterilization of explants

NaOCl and $HgCl_2$ both are oxidizing agents and damage the microorganism by oxidizing the enzymes. $HgCl_2$ is indicated 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) filed data of pepper which were sterilized by using different concentration of bavistin and mercuric chloride ($HgCl_2$). They showed that the best results in sterilization of nodal explants were recorded with using Bavistin 0.2% + 8-HQC (200ppm) for 1 hour. Jagatheeswari and Ranganathan (2012) found sterilization of explants with lower concentration of mercuric chloride treatments with short time and it's gave the best result. Pawar *et al*., (2015 suggested 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.

2.4. Shoot induction:

Haque *et al*. (2016) reported that an efficient for multiple shoot regeneration of *Capsicum* seeds germinated *in vitro* on a half-strength Murashige and Skoog (MS) medium supplemented with 3.0 % sucrose. The combination of the 6-benzylaminopurine, indole-3-acetic acid and spermidine was found to be the best for multiple shoot induction. However, the optimum response varied accompanied by different cultivars with maximum 8.9 ± 0.52 (Capsi-10) to 15.3 ± 0.69 (Capsi5) multiple shoot per explant.

Raj *et al*. (2015) observed that the multiple shoot of Naga King Chilli in multiple shoot was initially induced using MS medium supplemented with 5 mg/L BAP and 0.5 mg/L IAA or MS medium fortified with BAP

alone or in combination with IAA and adenine sulphate. Callus were then induced from the *in vitro* raised plants using stem segments by culturing on MS medium fortified with 3 mg/L BAP and 1mg/NAA.

Nowaczyk *et al*. (2015) studied that the propagation rate of *Capsicum* was significantly increased when IAA was added in a low concentration along with the optimum concentration of BAP.

Gayathri *et al*. (2015) developed an *in vitro* micropropagation protocol for *Capsicum chinense Jacq*. popularly known as habenaro pepper . The study revealed that MS medium supplemented with BAP alone or in combination with IAA, AdenineSulphate for multiple shoot development. Maximum numbers of shoot buds were produced in MS medium containing 19.98 μM BAP with 5.71 μM IAA and 13.31 μM BAP with 13.56 μM Ad.S. Successful induction of Callus from leaf and internodal part of *in vitro* raised plants were achieved in MS medium in combination of 6.66 μM BAP with 9.05 μM 2.4.D and 6.66 μM BAP with 8.06 μM NAA respectively.

Fatima *et al*. (2014) showed that maximum calli (83%) of chilli induced in cotyledon explant on MS medium containing 2, 4-D (1 mg/L) through *in vitro* plant regeneration. When sub-cultured on auxin free medium, no embryogenesis could be observed in calli. Highest shoot regeneration potential (53%) was observed in Cotyledon leaf explant induced calli on MS medium containing BAP and Kin (3 mg/L + 1 mg/L) using cotyledon as explant.

Rizwan *et al.(*2013) reported that the combinations of BA + IAA and BAP alone induced shoot regeneration and Higher shoot induction in nodes and shoot tip explants on MS-medium supplemented with higher dose of BAP 8 mg/L in MS-medium , BAP 0.3 mg/L alone and BAP 0.5 mg/L and Kin 1.5 mg/L.

Bustos *et al*., (2009) developed a reliable protocol for the *in vitro* regeneration of four types of chili, *Capsicum annuum var*. *annuum, C. annuum* var. glabriusculum aviculare (Piquin), and *C. chinense* (Habanero) by direct organogenesis using three different explants (cotyledon, hypocotyls and embryo). The highest *in vitro* regeneration efficiency (14.6 shoots per explant) was achieved when Habanero chili hypocotyls were grown on Murashige and Skoog medium containing 1.7 μM indole-3 3 acetic acid and 22.2 μM N 6-benzyladenine.

Sharma *et al*., (2008) conducted that an *in vitro* regeneration from cotyledon, leaf and hypocotyl explants of six cultivars belonging to three species of Capsicum was achieved by direct organogenesis. Leaf and cotyledon explants regenerated more shoots than hypocotyl explants and the maximum number of shoots were produced on Murashige and Skoog (1962) medium containing 8.8 μM 6-benzylaminopurine (BAP) with 11.4 μM indole-3-acetic acid (IAA). Elongation of shoot buds derived from different explants was achieved on medium containing 2.8 μM IAA and the elongated shoots were rooted on medium containing 2.8 or 5.7 μM IAA and 2.4 or 4.9 μM indole-3-butyric acid (IBA). Four-week old rooted plantlets were hardened and transplanted to the soil.

Stewart *et al*. (2007) reported that callus induced of Naga King Chilli from placental tissues using MS medium supplemented with 2mg/L 2,4-D (2,4 -dichlorophenoxyacetic acid) and 0.5 mg/L Kinetin producing a good amount of proliferation and friable callus. This finding is significant taking into consideration the fact that capsaicinoids are exclusively synthesized in chilli fruits, specifically in the placenta and the interlocular septum, where they accumulate in vesicles.

Hasnat *et al*. (2007) developed an efficient and reliable *In vitro* protocol for induction and regeneration of calli in varieties of hot chilli (*Capsicum frutescens* L.) var. Nepali and NARC-IV. Early initiation and induction with sustainable calli growth in both varieties was achieved on Murashige

& Skoog medium supplemented with 1.5 mg l-1 of 2, 4-D. Better performance in both varieties regarding shoot initiation, regeneration rate (%) and number of lateral shoots per regenerate were achieved on medium containing 3.0 mg l-1 BA. Rooting was achieved on half strength MS basal medium containing 1.0 mg/L IBA.

Sanatmbi *et al*. (2007) developed a novel micropropagation protocol for *Capsicum frutescens* L. cv. 'Uchithi', a pungent chilli cultivar, 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. 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-benzylaminopurine (BAP). Maximum number of shoots (5.6) were induced on medium containing 22.2 mM BAP 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).

Kumar *et al*. (2005) showed that optimal concentration of 2,4-D maintained maximum growth (26-29 calli) due to increased DNA and RNA synthesis with accelerated growth.

Auer *et al*. (2002) showed that 3.0 mg l-1 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.

Christopher and Rajan, (2000) showed that very high levels of BAP (66.6–88.8 mM) and kinetin (92.9–116.2 mM) were necessary for maximal shoot proliferation from shoot-tip explants of Capsicum. However, in the present study, the number of shoot buds regenerated was reduced when the concentration of BAP 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. The best rooting and elongation occurred on medium containing 2.8 mM IAA or 2.4 and 4.9 mM IBA.

2.5. 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. Christopher *et al*. (1994) used 2,3,5-tri iodo benzoic acid (TIBAP), an inhibitor of auxin transport, with a low concentration of BAP or kinetin to assess its effect on shoot proliferation. They suggested that TIBAP 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.

Franck Duchenne *et al*. (1998) used a plant steroid lactone 24-epi-brassinolide (EBR) (0.02 mg/L) in MS medium and found that EBR did not always act directly on stem elongation in concert with exogenous and endogenously added growth regulator.

Zhu *et al*. (l996) developed that the increase the number of elongated shoots. In the first step, multiple buds obtained from cotyledonary leaves were cultured on MS + 2 mg/L BAP + 2 mg/L GA3 for 10 days and then transferred to MS + 2 mg/L GA3 + 0.5 mg/L BAP developed about 10 mature plants from a single explant.

Ochoa- Alejo *et al*. (1990) conducted that a new method for *in vitro* chilli plant regeneration based on shoot formation from wounded hypocotyls. Seedlings at the stage of curved hypocotyls wounded in the apical region of hypocotyls were the best explants for shoot regeneration when inoculated on culture medium without growth regulators.

A highly efficient three stage protocol for the regeneration of chilli pepper (*Capsicum annuum* L.) 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 (5 or 7 mg/L) + PAA (2 mg/L). Buds were elongated during the second stage on medium containing BAP (2 to 5 mg/L) + PAA (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 BAP + PAA. The shoots that elongated in the second stage rooted at 100 per cent frequency on a medium supplemented with NAA (1 mg/L).

2.6. Root induction

Ediba *et al*.(2001) examined that the combined effect of 0.5 mg/L lAA and 0.4 mg/L NAA on root induction was demonstrated by Ediba and Hu (1993). Kim *et al*. (2001) observed that the optimal rooting condition in MS with 0.3 mg/L NAA.

Christopher *et al*. (1986) reported that in tissue cultures of chilli on the shoot induction medium normally roots are not induced. Therefore, rooting has to be induced by sub-culturing on medium with auxins. Agarwal et al. (2001) observed that root induction by sub-culturing the shoots on MS medium with lAA (1 mg/L) and kinetin (0.1 mg/L) was reported by MS medium with 0.1 mg/L of IBA was used for root induction. Depending on the cultivar, multiplied shoots were successfully rooted with maximum 18.4 ± 0.20 (highest for Capsi-9) to 36.8 ± 0.29 (highest for Capsi-5) roots per shoot on half-strength MS medium

supplemented with 2.0 mg/L indole-3-butyric acid, 1.0 mg/L α-naphthalene acetic acid, and 1.5 mM spermidine. Finally, the micropropagated plantlets were acclimatized with 40.0–86.7 % survival rate, depending on different cultivars.

2.7. Hardening and establishment of the regenerated plants

Yapu *et al*. (2011) transferred regenerated plantlets of pineapple on to specially made polyethylene bags containing soil mixture and moved those to the greenhouse and hefound that survival rate of the plantlets under natural conditions was 98% and maximum average number of plantlets (80 ± 0.6). The well-developed plantlets were transferred to an open field where the plants produced normal fruits.

Nualbunruang and Chidburee (2004) used seven different media to observe significant impact on some selected parameters of vegetative growth of pineapples. In general, mixture of sand :coconut-compost :charcoal husk (1:1:1), sand :charcoal husk (1:1), soil :sand :charcoal husk (1:1:1) and soil :sand :charcoal husk (1:1:1) gave higher values of growth parameter such as number of leaves per plant, plant height, leaf length and leaf width in comparison to other media ($p<0.05$).

CHAPTER III

MATERIALS AND METHODS

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 (SAU), Sher-e-Bangla Nagar, Dhaka-1207 from the period of February, 2017 to January, 2018.

3.2 Experimental materials

3.2.1 Source of materials

The healthy explants were collected from Bangladesh Agricultural Research Institute(BARI), Gazipur and Horticultural Farm of Sher-e-Bangla Agricultural University(SAU), Dhaka-1207 and different part of the country.

3.2.2 Plant materials

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

Plate 1. Explants collection from *Capsicum annuum* L.

3.2.3. Small instruments and glass ware

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

3.2.4. Culture media

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

3.3. Sterilization of small instruments and Glass ware

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

3.4. Preparation of stock solutions

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

3.4.1. Stock solution A (Macronutrients)

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

3.4.2. Stock solution B (Micronutrients)

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

3.4.3. Stock solution C (Iron sources)

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

3.4.4. Stock solution D (Vitamins)

Each of the desired ingredients except myo-inositol were taken at 10 folds (100x) of their final strength in a measuring cylinder and dissolved in 750 ml of distilled water. Then the final volume was made up to 1000 ml by further addition of distilled water. The solution was dispensed into 10 ml aliquots and stored at (4^0C). Myo-inositol was used directly at the time of media preparation.

3.4.5. Plant growth regulators (PGRs)

Following stock solutions of hormones were prepared ahead of media preparation and stored at 4ºC temperature.

1. BAP (1.0, 2.0, 3.0 and 4.0 mg/L) for shoot induction
2. BAP (1.0, 2.0, 3.0 and 4.0 mg/L) combination with IBA (1.0, 1.5, 2.0 and 2.5 mg/L) respectively for callus and shoot induction
3. IBA (1.0, 1.5, 2.0 and 2.5 mg/L) for root formation

3.5. Preparation of the stock solution of hormones

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

Hormone (solute)	Solvents used
BAP	1N NaOH
IBA	70% ethyl alcohol

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

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

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

3.6. Preparation of culture media from ready made MS powder

To prepare 1 litre of MS (Murashige and Skoog, 1962) medium the following steps were followed:

1. About 400 ml distilled water was taken in a flask.
2. 100 ml of macronutrients, 10 ml of micronutrients, 100 ml of irons and 10 ml of vitamins were taken from each of these stock solutions into a 2 litre beaker on a heater cum magnetic stirrer.
3. 30 gm of sucrose was added to this solution and gently agitated to dissolve completely.
4. Required volume of hormone solutions was directly added to the solutions in the beakers.
5. The solution was poured into a 1000 ml measuring cylinder and the volume was made up to 1000 ml with addition of distilled water.
6. pH of the medium was adjusted to 5.8 with a digital pH meter with the help of adding 0. 1 N NaOH or 0.1 N HCl as necessary.

7. To solidify the medium, 8 gm (0.8%) of Difco-brand Bacto-Agar was added to the solution and the whole mixture was heated (avoiding boiling) by micro oven for 5 to 10 minutes till complete dissolution of the agar.

8. 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 done 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 21±2 °C for several hours to make it ready for inoculation with explants.

3.8. Sterilization of culture room and transfer area

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

The transfer area was also cleaned with detergent and also sterilized twice in a month by 70% ethanol. Laminar airflow cabinet was usually sterilized by switching UV ray to kills the microbes inside the laminar airflow. It switches on 30 minutes before working in empty condition and for 20 minutes with all the instruments. The working surface was wiping with 70% ethanol before starting the transfer work.

3.9 Explants preparation and culture

3.9.1 Preparation of explants

The shoot tip and nodal segments was used as explants. It was obtained from developing shoots of pepper grown under field conditions and was brought to the

preparation room .The shoot tip and nodal segments was washed with tap water in a beaker. The shoot tip then cut in 0.5-1.0 cm size required for inoculation in culture vial.

3.9.2 Surface sterilization of explants

The shoot tip and of 2 to 3 cm size were taken in a beaker. Surface sterilization of explants was done as follows:

i. The shoot tip were cut as small size (0.5 to 1.0cm) and rinsed with water for several times.

ii. Washing with distilled water was done for several times.

iii. Then explants were sterilized with 70% ethanol for 1 min, followed by 3 to 4 times rinsing with distilled water.

iv. After that the explants were sterilized with 0.1% $HgCl_2$ adding few drops of tween-20 for 5 min.

v. Then explants were rinsed with sterilized distilled water for at least 4 times.

vi. vii. Finally the explants were transferred to the MS media carefully

3.10 Culture of explants

3.10.1 Inoculation of explants in culture vial

The isolated and surface sterilized shoot tip was collected carefully through maintaining aseptic condition inside the laminar air flow cabinet. The individual shoot tip were directly inoculated to each of the culture vial containing 25 mL of MS medium supplemented with different concentrations of hormones as per treatment. Explants were transferred to culture vials with the help of sterile forceps under strict aseptic conditions. 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 explant on the medium (Plate 2).

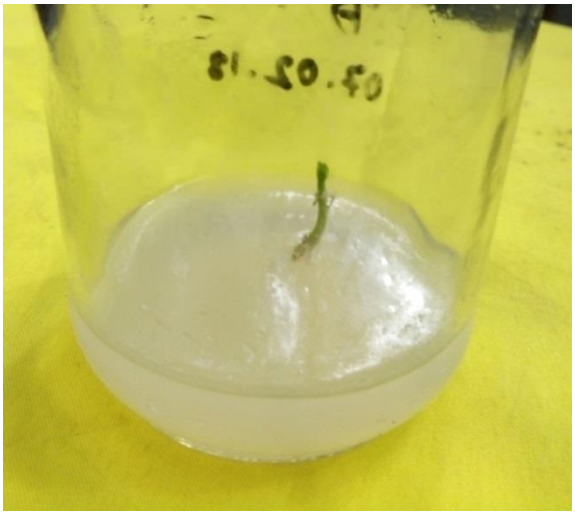

Plate 2. Inoculation of explant in culture vial

3.10.2 Incubation

The culture vials transferred to culture racks and allowed to grow in controlled environment. The temperature of the growth room was maintained within 21±1ºC by an air conditioner and a 16 hour photo period was maintained along with light intensity of 3000-5000 lux for proper growth and development of culture.

3.10.3 Maintenance of proliferating shoots

Initial sub-culturing was done after 20-25 days when the explants had produced some shoots. For sub-culturing, the entire samples of *in vitro* shoot were cut into small pieces 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 10-20 days.

3.10.4 Root induction of regenerated shoots

When the shoots grew about 3-4 cm in length with 4-5 well developed leaves, they were removed aseptically from the culture vials and were separated from each other and again cultured on freshly prepared medium containing different combinations of hormonal supplements for root induction.

3.11 Treatments

Four sub-experiments were conducted to assess the effect of different concentrations of BAP and IBA on shoot proliferation and subsequent rooting of the multiplied shoot.

Sub-experiment 1.

Effect of BAP on *in vitro* shoot induction potentiality in *Capsicum annum L.*

Four levels of BAP (1.0, 2.0, 3.0 and 4.0 mg/L) and control (0.0 mg/L) were use. The experiments were arranged in Completely Randomized Design (CRD) with four replications.

Sub-experiment 2.

Combined effect of BAP and IBA on *in vitro* callus and shoot induction in *Capsicum annum* L.

In this sub-experiment, four levels of IBA were practiced with each level of BAP (1.0, 2.0, 3.0 and 4 mg/L). Total 16 combinations of BAP and IBA were examined in this experiment and control treatment also practiced. The combinations were as follows:

T1 = BAP 1.0 mg/L + 1.0 mg/L IBA
T2 = BAP 1.0 mg/L + 1.5 mg/L IBA
T3 = BAP 1.0 mg/L + 2.0 mg/L IBA
T4 = BAP 1.0 mg/L + 2.5 mg/L IBA
T5 = BAP 2.0 mg/L + 1.0 mg/L IBA
T6 = BAP 2.0 mg/L + 1.5 mg/L IBA
T7 = BAP 2.0 mg/L + 2.0 mg/L IBA
T8 = BAP 2.0 mg/L + 2.5 mg/L IBA
T9 = BAP 3.0 mg/L + 1.0 mg/L IBA
T10 = BAP 3.0 mg/L + 1.5 mg/L IBA
T11 = BAP 3.0 mg/L + 2.0 mg/L IBA
T12 = BAP 3.0 mg/L + 2.5 mg/L IBA
T13 = BAP 4.0 mg/L + 1.0 mg/L IBA
T14 = BAP 4.0 mg/L + 1.5 mg/L IBA
T15 = BAP 4.0 mg/L + 2.0 mg/L IBA
T16 = BAP 4.0 mg/L + 2.5 mg/L IBA

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

Sub-experiment 3.

Effect of IBA on root induction potentiality of *in vitro* regenerated *Capsicum annum*

Four levels of IBA (1.0, 1.5, 2.0 and 2.5 mg/L) and control (0.0 mg/L) treatment were used. The experiments also practiced as sub-experiment 1.

Sub-experiment 4.

Acclimatization and establishment of plantlets on soil

3.12. 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 14, 21 and 35 days of culture, starting from day of inoculation on culture media in case of shoot proliferation. In event of root formation, it was done every week starting from third week to fifth week of culture.

The following observations were recorded in cases of shoot and root formation under *in vitro* condition.

1. Days for shoot induction
2. Days to callus induction
3. Days to initiate shoot from callus
4. No. of shoots per explants
5. Length of shoot (cm)
6. Days to root induction
7. No. of roots per explants
8. Length of root (cm)
9. Percent of explants showing shoot induction
10. Percent of explants showing root induction

3.12.1. Days to callus induction

Generally, callus induction started after few days of explants incubation. Days to callus induction was recorded when it was initiated from explants. The mean value of data provided the days to callus induction.

3.12.2. Calculation of days to shoot induction from callus

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

3.12.3. Calculation of percent of shoots and root induction from culture

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

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

The percentage of root induction was calculated as:

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

3.12.4. Calculation of shoot and root length (cm)

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

3.12.5. 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-

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

3.12.6. Number of leaf

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

3.13. Acclimatization

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

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

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

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

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

3.14. Statistical analysis of data

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 five 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 callus induction, shoot regeneration, root induction and establishment of plantlets in the field condition. Major findings were presented in different sub headings.

4.1. Sub-experiment 1. Effect of BAP on *in vitro* shoot induction of *Capsicum annum* L.

The result of different concentrations of BAP has been presented under following headings with Figure, Plate and Table.

4.1.1 Days to shoot induction

Significant variations were observed among different concentration of BAP on days to shoot induction. The maximum 36.25 days to shoot induction were recorded in control treatment followed by 1.0 mg/L (27.25 days) and 4.0 mg/L (23.75 days). On the other hand, minimum 18.25 days were required in 3.0 mg/L BAP followed by 2.0 mg/L (22.75 days) (Figure 1).

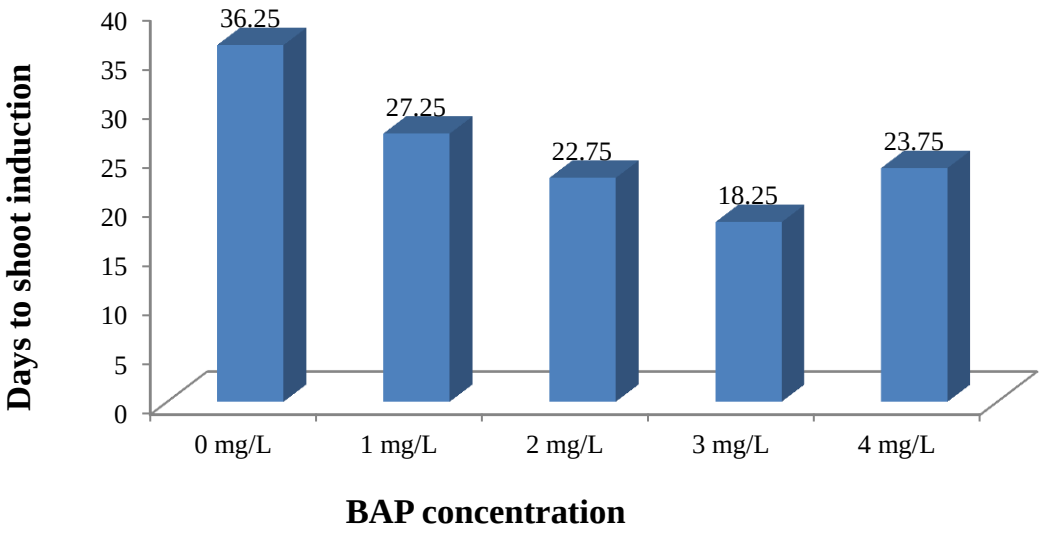

Figure 1. Effect of BAP on days to shoot induction

4.1.2 Percent of shoot induction

There was significant variation on percent of shoot induction at different concentration of BAP. The highest percentage (72%) of shoot induction was induced in the treatment 3.0 mg/L BAP and the lowest percentage (37%) was induced in hormone free media (Figure 2).

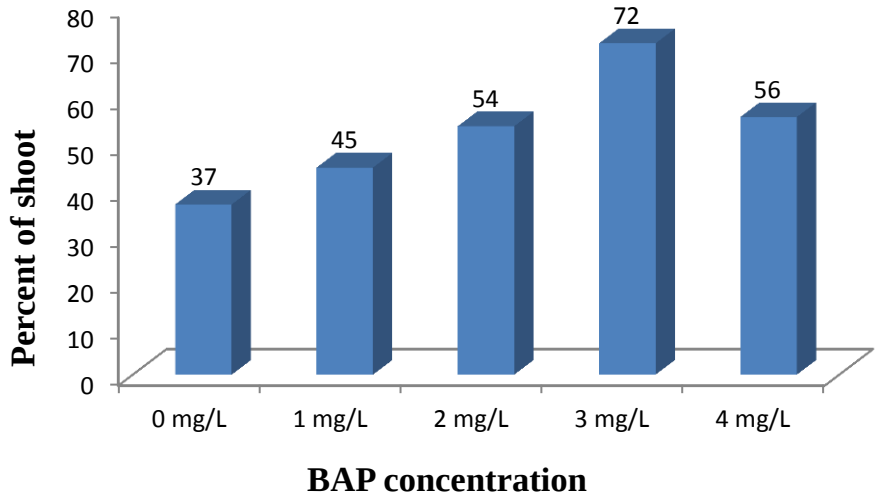

Figure 2. Effect of BAP on percent of shoot induction

4.1.3 Number of shoot

There was a significant influence of different concentrations of BAP on the number of shoot at 5% level of significance in laboratory condition. The highest number of shoots (1.75, 2.75 and 3.50) at 14, 21 and 35 DAI, respectively were noticed from the 3.0 mg/L (Table 1). Whereas the lowest number of shoots (1.00, 1.50 and 2.15) at 14, 21 and 35 DAI, respectively were noticed in control treatment (Table 1). Haque *et al*. (2016) reported maximum 8.9 ± 0.52 (Capsi-10) to 15.3 ± 0.69 (Capsi5) multiple shoot per explant in combination of the 6-benzylaminopurine, indole-3-acetic acid and spermidine in *Capsicum* sp. The findings of this study are partially similar with their results.

Table 1. Effect of different concentration of BAP on the number of shoot at different days after shoot induction

BAP (mg/L)	Number of shoot		
	14 DAI	21 DAI	35 DAI
0	1.0 d	1.50 d	2.15 d
1.0	1.15 c	1.75 c	2.20 c
2.0	1.25 b	2.00 b	2.55 c
3.0	1.75 a	2.75 a	3.50 a
4.0	1.25 b	2.20 b	2.25 b
CV%	21.89	14.43	11.32
LSD(0.05)	1.18	1.28	1.48

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 (cm)

There was a significant influence of different concentrations of BAP on the length of shoots. The treatment BAP 3.0 mg/L gave the maximum length of shoots (2.05, 3.13 and 4.83) (Plate 3) and the second highest shoot length (1.78, 2.50 and 3.60) was found in BAP 4.0 mg/L at 14, 21 and 35 DAI, respectively (Table 2) and (plate 3) whereas, the control treatment showed the lowest length of shoots (0.75, 1.25 and 2.15) at 14, 21 and 35 DAI (Table 2) respectively. Cucel *et al.* (2017) found that the highest shoot multiplication successes were obtained in the lowest BAP treatments with 38.88 mm shoot length of chilli. The variation may be due to the influence of genetic and environmental factors (Sen, J. *et al*., 2002).

Plate 3. Highest length of shoot at 35 DAI in the treatment 3.0 mg/L BAP

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

BAP (mg/L)	Shoot Length		
	14 DAI	21 DAI	35 DAI
0	0.75 c	1.25 c	2.15 c
1.0	1.48 b	2.40 b	3.00 b
2.0	1.58 ab	2.38 b	3.43 b
3.0	2.05 a	3.13 a	4.83 a
4.0	1.78 ab	2.50 ab	3.60 ab
CV%	8.62	11.92	16.66
LSD(0.05)	0.33	0.68	1.37

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.

4.1.5. Number of leaves per shoot

There was a significant influence of different concentrations of BAP on the number of leaves per shoot. BAP 3.0 mg/L gave the maximum number of leaves (3.50, 6.00 and 7.50) (Plate 4) and the second highest leaves number (2.75, 4.50 and 6.25) was found in BAP 4.0 mg/L at 14, 21 and 35 DAI, respectively (Table 3) whereas, the control treatment showed the lowest number of leaves (1.00, 1.53 and 2.15) at 14, 21 and 35 DAI, (Table 3) respectively.

Plate 4. Highest number of leaves at 35 DAI in the treatment 3.0 mg/L BAP

Table 3. Effect of different concentration of BAP on leaf number at different DAI

BAP (mg/L)	Leaf number		
	14 DAI	21 DAI	35 DAI
0	1.00 c	1.53 e	2.15 e
1.0	1.50 b	2.50 d	3.50 d
2.0	2.50 ab	3.50 c	5.00 c
3.0	3.50 a	6.00 a	7.50 a
4.0	2.75 ab	4.50 b	6.25 b
CV%	19.18	10.69	10.67
LSD(0.05)	1.18	0.97	1.47

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. Sub Experiment 2. Effect of BAP and IBA on callus induction in *Capsicum annum L.*

This experiment was conducted under laboratory condition to evaluate the effect of BAP and IBA on callus induction and multiple shoot induction in bell pepper. The results are presented separately under different headings below.

4.2.1. Days to callus induction

Significant variations were observed among different concentrations of BAP and IBA on days to callus induction. The minimum duration 16.00 days was obtained in BAP 2.0 mg/L+ IBA 1.5 mg/L than rest of the treatments. On the other hand, the maximum days 34.25 days to callus induction was recorded in BAP 1.0 + IBA 1.0 mg/L (Table 4).

4.2.2. Percentage of callus induction

There was a significant influence of different concentrations of BAP and IBA on the percentage of callus formation per explant. The optimum percentage (78.00%) of callus formation was noticed in treatment BAP 3.0 mg/L + IBA 2.0 mg/L which was best than others and minimum percentage (35.00%) was noticed in BAP 1.0 + IBA 1.0 mg/L(Table 4). Fatima *et al*. (2014) showed that maximum calli (83%) of chili induced in cotyledon explant on MS medium containing 2, 4-D (1 mg/L) through *in vitro* plant regeneration. This variation due to different growth regulators in the culture media and genetical, physiological and morphological variation *in vitro* (Narayanswamy *et al*., 1977).

Table 4. Combined effect of BAP and IBA on days to callus induction and percent of callus induction

Treatments	Callus induction days	Percent of (%) callus induction
BAP 1.0 + IBA 1.0 mg/L	34.25 a	37 g
BAP 1.0 + IBA 1.5 mg/L	22.33 b-e	42 fg
BAP 1.0 + IBA 2.0 mg/L	18.66 de	45 f
BAP 1.0 + IBA 2.5 mg/L	19.66 de	48 f
BAP 2.0 + IBA 1.0 mg/L	18.66 de	52 ef
BAP 2.0 + IBA 1.5 mg/L	16.00 e	54 de
BAP 2.0 + IBA 2.0 mg/L	20.66 c-e	62 cd
BAP 2.0 + IBA 2.5 mg/L	23.00 b-d	54 de
BAP 3.0 + IBA 1.0 mg/L	23.33 b-d	65 bc
BAP 3.0 + IBA 1.5 mg/L	28.67 ab	68 bc
BAP 3.0 + IBA 2.0 mg/L	24.00 b-d	78 a
BAP 3.0 + IBA 2.5 mg/L	24.33 b-d	65 bc
BAP 4.0 + IBA 1.0 mg/L	25.00 a-d	55 cd
BAP 4.0 + IBA 1.5 mg/L	28.33 ab	56 cd
BAP 4.0 + IBA 2.0 mg/L	26.66 a-c	58 d
BAP 4.0 + IBA 2.5 mg/L	31.33 ab	54 de
CV (%)	8.96	22.23
$LSD_{(0.05)}$	6.34	7.15

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.

4.2.3. Callus size

The highest callus size (1.20 cm, 2.23 cm and 3.26 cm) at 14, 21 and 35 DAI, respectively was found in BAP 3.0mg/L + IBA 2.0mg/L (plate 5) which is statistically similar with BAP 3.0mg/L+ IBA 1.5mg/L (1.03 cm, 2.10 cm and 3.0 cm) at 14, 21 and 35 DAI, respectively. (Table 5). The lowest callus size (0.56 cm, 1.10 cm and 1.74 cm) at 14, 21 and 35 DAI (Table 5) was found in the treatment of BAP 1.0mg/L + IBA 1.0 mg/L.

Plate 5. Callus size at 35 DAI in the treatment 3.0 mg/L BAP + IBA 2.0mg/L.

Table 5. Combined effect of different concentration of BAP and IBA on callus size at different DAI

Treatments	Callus size		
	14 DAI	21 DAI	35 DAI
BAP 1.0 + IBA 1.0 mg/L	0.56 e	1.10 e	1.74 h
BAP 1.0 + IBA 1.5 mg/L	1.00 de	1.40 de	2.00 gh
BAP 1.0 + IBA 2.0 mg/L	1.36 cd	1.76 b-e	2.23 fg
BAP 1.0 + IBA 2.5 mg/L	1.16 c-e	1.36 e	1.86 h
BAP 2.0 + IBA 1.0 mg/L	1.20 c-e	1.53 c-e	2.26 e-g
BAP 2.0 + IBA 1.5 mg/L	1.36 cd	1.66 c-e	2.36 d-f
BAP 2.0 + IBA 2.0 mg/L	1.53 bc	1.90 b-e	2.30 d-f
BAP 2.0 + IBA 2.5 mg/L	1.20 c-e	1.70 c-e	2.23 fg
BAP 3.0 + IBA 1.0 mg/L	1.17 bc	2.23 bc	2.60 bc
BAP 3.0 + IBA 1.5 mg/L	1.13 bc	2.06 b	2.60 bc
BAP 3.0 + IBA 2.0 mg/L	1.20 a	2.23 a	3.26 a
BAP 3.0 + IBA 2.5 mg/L	1.53 bc	2.03 b-d	3.13 b
BAP 4.0 + IBA 1.0 mg/L	1.16 c-e	1.43 c-e	2.40 d-f
BAP 4.0 + IBA 1.5 mg/L	1.03 cd	1.56 b-e	2.50 cd
BAP 4.0 + IBA 2.0 mg/L	1.10 bc	2.10 bc	3.00 b
BAP 4.0 + IBA 2.5 mg/L	1.23 c-e	1.90 b-e	2.50 d-f
CV (%)	11.91	12.96	8.37
$LSD_{(0.05)}$	0.51	0.75	0.35

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. Combined effect of BAP and IBA on days to shoot initiation from callus

Significant variations were observed among different concentrations of BAP and IBA on days to shoot induction from callus. The minimum duration 17.00 days was obtained in BAP 3.0 mg/L + IBA 2.0 mg/L than rest of the treatments. On the other hand, the maximum days 35.25 days was recorded in treatment BAP 1.0 mg/L + IBA 1.0 mg/L (Table 6). Daud *et al*. (2015) showed that the new plantlets were raised in a short period of time when explants were cultured on MS medium containing 1.0 mg/1 BAP and 0.5 mg/1 NAA in chilli. The variation may be due to influence of genetic and environmental factors (Sen, J. *et al*., 2002).

4.2.5. Percentage of shoot induction from callus

There was a significant influence of different concentrations of BAP and IBA on the percentage of shoot induction from callus. The optimum percentage (75.00%) of shoot induction was noticed in treatment BAP 3.0 mg/L + IBA 2.0 mg/L which was best than others and minimum percentage (32.15%) was noticed in treatment BAP 1.0 mg/L + IBA 1.0 mg/L (Table 6). Abbasi *et al.* (2016) observed that the treatment 5.0 mg/L BAP and 1.0 mg/L NAA produced optimum percentage of shoot organogenesis after 4 weeks of sub-culturing. Due to different growth regulators and genetical, physiological and morphological factors are responsible for the above variation (Narayanswamy, S. *et al*., 1977).

Table 6. Combined effect of BAP and IBA on days to shoot induction and percent of shoot induction from callus

Treatments	Shoot induction	Percent of (%) shoot induction
BAP 1.0 + IBA 1.0 mg/L	35.25 a	37 g
BAP 1.0 + IBA 1.5 mg/L	28.33 ab	41 fg
BAP 1.0 + IBA 2.0 mg/L	24.33 b-d	42 f
BAP 1.0 + IBA 2.5 mg/L	26.66 a-c	47 f
BAP 2.0 + IBA 1.0 mg/L	28.67 ab	53 ef
BAP 2.0 + IBA 1.5 mg/L	24.00 b-d	54 de
BAP 2.0 + IBA 2.0 mg/L	20.66 c-e	62 cd
BAP 2.0 + IBA 2.5 mg/L	23.00 b-d	55 de
BAP 3.0 + IBA 1.0 mg/L	23.33 b-d	65 bc
BAP 3.0 + IBA 1.5 mg/L	19.66 de	66 bc
BAP 3.0 + IBA 2.0 mg/L	17.00 e	75 a
BAP 3.0 + IBA 2.5 mg/L	18.66 de	66 bc
BAP 4.0 + IBA 1.0 mg/L	25.00 a-d	54 cd
BAP 4.0 + IBA 1.5 mg/L	22.33 b-e	55 cd
BAP 4.0 + IBA 2.0 mg/L	18.66 de	58 d
BAP 4.0 + IBA 2.5 mg/L	21.667	54 de
CV (%)	8.96	22.23
$LSD_{(0.05)}$	6.34	7.15

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.

4.2.6 Number of shoot

The highest number of shoot (2.40, 2.23 and 3.76) was noticed from the BAP 3.0 mg/L + IBA 2.0 mg/L (Plate 6) and second highest number (1.56, 2.23 and 3.16) at 14, 21 and 35 DAI, respectively, were observed in 4.0 mg/L BAP + 2.0 mg/L IBA. Whereas the lowest average number of shoot (0.73, 1.23 and 1.80) at 14 DAI, 21 DAI and 35 DAI, respectively were noticed in treatment BAP 1.0 mg/L + IBA 1.0 mg/L (Table 7). These findings are completely opposed 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 6-benzylaminopurine (BAP) in combination with 2.85 µM indole-3-acetic acid (IAA). This variation is due to the age, nature, origin and the physiological state of the explant and environmental factors can play a crucial role in the establishment of cultures and subsequent plant regeneration (Bajaj, Y.P.S. *et al*., 1991).

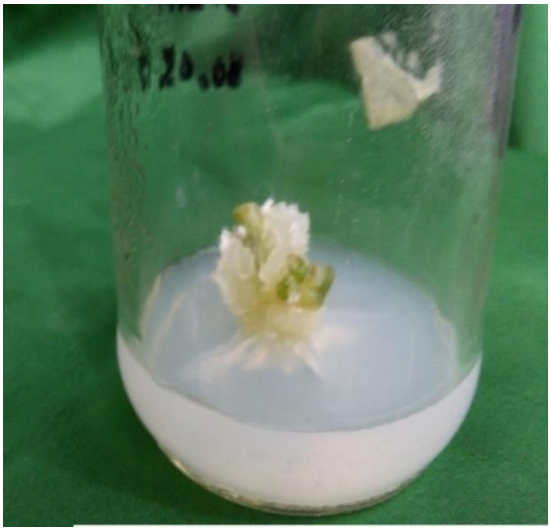

Plate 6. Highest number of shoot at 35 DAI in the treatment BAP 3.0 mg/L + IBA 2.0 mg/L

Table 7. Combined effect of different concentration of BAP and IBA on number of shoot at different DAI

Treatments	Number of shoot		
	14 DAI	21 DAI	35 DAI
BAP 1.0 + IBA 1.0 mg/L	0.73 e	1.23 e	1.80 h
BAP 1.0 + IBA 1.5 mg/L	1.00 de	1.40 de	2.00 gh
BAP 1.0 + IBA 2.0 mg/L	1.36 cd	1.76 b-e	2.23 fg
BAP 1.0 + IBA 2.5 mg/L	1.16 c-e	1.36 e	1.86 h
BAP 2.0 + IBA 1.0 mg/L	1.20 c-e	1.53 c-e	2.26 e-g
BAP 2.0 + IBA 1.5 mg/L	1.36 cd	1.66 c-e	2.36 d-f
BAP 2.0 + IBA 2.0 mg/L	1.53 bc	1.90 b-e	2.50 d-f
BAP 2.0 + IBA 2.5 mg/L	1.20 c-e	1.70 c-e	2.23 fg
BAP 3.0 + IBA 1.0 mg/L	1.52 bc	2.23 bc	2.60 de
BAP 3.0 + IBA 1.5 mg/L	1.93 ab	2.46 b	3.00 bc
BAP 3.0 + IBA 2.0 mg/L	2.40 a	3.23 a	3.76 a
BAP 3.0 + IBA 2.5 mg/L	1.53 bc	2.13 b-d	3.23 b
BAP 4.0 + IBA 1.0 mg/L	1.16 c-e	1.63 c-e	2.40 d-f
BAP 4.0 + IBA 1.5 mg/L	1.33 cd	1.96 b-e	2.70 cd
BAP 4.0 + IBA 2.0 mg/L	1.56 bc	2.23 bc	3.16 b
BAP 4.0 + IBA 2.5 mg/L	1.23 c-e	1.90 b-e	2.50 d-f
CV (%)	11.91	12.96	8.37
$LSD_{(0.05)}$	0.51	0.75	0.35

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.

4.2.7. Length of shoot

The maximum length of shoot (1.15, 2.33 and 4.53 cm) was noticed from the BAP 3.0 mg/L + IBA 2.0 mg/L (Plate 7) and second highest length(1.02, 2.13 and 4.03 cm) at 14, 21 and 35 DAI, respectively, were observed in 4.0 mg/L BAP + 2.0 mg/L IBA. Whereas the lowest average number of shoot (0.56, 1.22 and 1.75 cm) at 14 DAI, 21 DAI and 35 DAI, respectively were noticed in control treatment (Table 8).

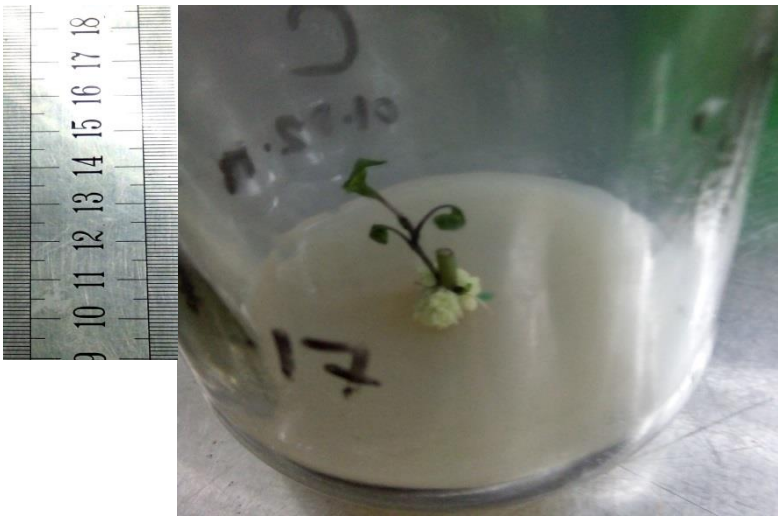

Plate7. Length of shoot at 35 DAI in the treatment BAP 3.0 mg/L + IBA 2.0 mg/L

Table 8. Combined effect of different concentration of BAP and IBA on length of shoot at different DAI

Treatments	Shoot Length		
	14 DAI	**21 DAI**	**35 DAI**
Control	00	00	00
BAP 1.0 + IBA 1.0 mg/L	0.56 c	1.22 d	1.75 c
BAP 1.0 + IBA 1.5 mg/L	0.67 bc	1.34 b-d	1.87 c
BAP 1.0 + IBA 2.0 mg/L	0.76 bc	1.43 b-d	1.95 c
BAP 1.0 + IBA 2.5 mg/L	0.86 a-c	1.60 a-d	3.00 b
BAP 2.0 + IBA 1.0 mg/L	1.06 bc	1.60 cd	3.36 b
BAP 2.0 + IBA 1.5 mg/L	1.13 a-c	2.13 a-d	3.53 ab
BAP 2.0 + IBA 2.0 mg/L	1.00 bc	2.33 a-d	3.43 ab
BAP 2.0 + IBA 2.5 mg/L	1.10 a-c	1.96 a-d	3.23 b
BAP 3.0 + IBA 1.0 mg/L	1.00 bc	2.00 a-d	3.70 ab
BAP 3.0 + IBA 1.5 mg/L	1.00 bc	2.34 a-d	3.75 ab
BAP 3.0 + IBA 2.0 mg/L	1.15 a	2.33 a	4.53 a
BAP 3.0 + IBA 2.5 mg/L	1.10 ab	2.23 a-c	3.98 ab
BAP 4.0 + IBA 1.0 mg/L	0.73 bc	1.75 b-d	3.90 ab
BAP 4.0 + IBA 1.5 mg/L	1.00 a-c	2.05 a-d	4.00 ab
BAP 4.0 + IBA 2.0 mg/L	1.02 a-c	2.13 a-d	4.03 ab
BAP 4.0 + IBA 2.5 mg/L	0.73 bc	2.11 a-d	3.83 ab
CV (%)	25.45	18.07	19.37
$LSD_{(0.05)}$	1.67	2.65	3.18

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. Sub Experiment 3. Effect of IBA on root induction in *Capsicum annum L.*

4.3.1. Days to root induction

Significant variations were observed among different concentration of IBA on days to root induction. The maximum (32.25 days) to root induction were recorded in control treatment followed by 1mg/L (25.35 Days) and 1.5 mg/L (20.75 days) .On the other hand, minimum (16.25 days) was recorded in 2.0 mg/L IBA followed by 2.5 mg/L (17.75 days) Figure (3).

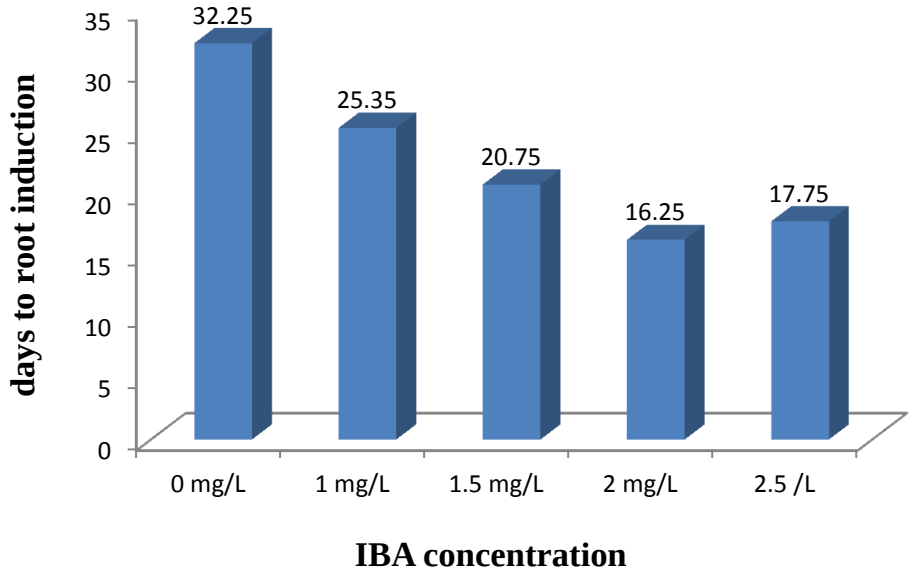

Figure 3: Effect of IBA on days to root induction

4.3.2 Percentage of root induction

There was significant variation on percent of explant showing root induction at different concentration of IBA. The highest percentage (82%) of root induction was induced in treatment 2.0 mg/L IBA and the lowest percentage (35%) was induced in hormone free media, Figure (4).

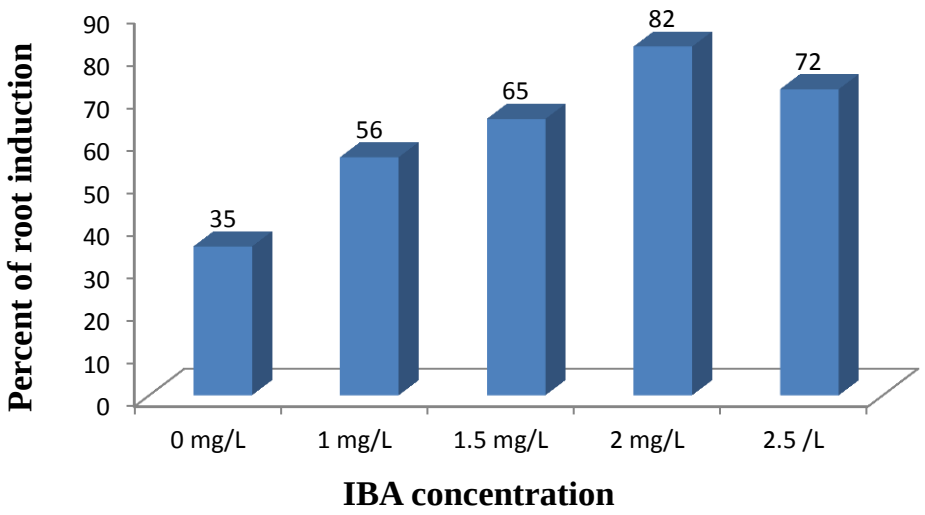

Figure 4: Effect of IBA on percent of root induction

4.3.3 Number of root per shoot

The highest number of root (3.25, 12.00 and 20.32) at 14, 21 and 35 DAI respectively (Plate 8) was noticed from the 2.0 mg/L of IBA (Table 9). Whereas the lowest number of root (1.10, 1.40 and 4.10) at 14, 21 and 35 DAI, respectively were noticed in control hormone free (Table 9).

Table 9. Effect of different concentration of BAP on the number of root at different DAI

IBA (mg/L)	Root Number		
	14 DAI	21 DAI	35 DAI
0	1.10 d	2.40 d	4.10 d
1.0	2.15 c	6.75 c	10.20 c
1.5	2.35 b	8.00 b	12.55 c
2.0	3.25 a	12.00 a	20.32 a
2.5	2.20 b	11.20 b	14.25 b
CV%	21.89	14.43	11.32
LSD(0.05)	1.18	1.28	1.48

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. Highest number of root at 35 DAI in the treatment 2.0 mg/L IBA

4.3.4 Length of root (cm)

There was a significant influence of different concentrations of IBA on the length of roots. IBA 1.5 mg/L gave the maximum length of roots (2.15, 2.75 and 4.25) (Plate 9) and the second highest root length (1.55, 2.43 and 3.13) was found in IBA 2.0 mg/L at 14, 21 and 35 DAI, respectively (Table 10) and (plate

9) whereas, the control treatment showed the lowest length of roots (0.36, 0.86 and 1.15) at 14, 21 and 35 DAI (Table 10) respectively.

Table 10. Effect of different concentration of IBA on the length of root at different DAI

IBA (mg/L)	Root Length(cm)		
	14 DAI	21 DAI	35 DAI
0	0.36 c	0.86 d	1.15 d
1.0	1.48 b	2.20 b	3.00 b
1.5	2.15 a	2.75 a	4.25 a
2.0	1.55 b	2.43 b	3.13 b
2.5	0.78 c	1.75 c	2.60 c
CV%	9.62	15.92	18.66
LSD(0.05)	0.43	0.78	1.77

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 35 DAI in the treatment 2.0 mg/L IBA

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

After two and half months, a good number of root and shoot were found and plants were acclimatized under shade condition and in natural condition. Twenty plants were transplanted in shade area after three weeks, 16 plantlets were survived and survival rate was 80% (Table 11). In natural condition, 16 plants were transplanted, 12 survived (Plate 10) and survival rate was 75%. Sharma *et al.* (2012) found that plantlets of *Capsicum annum* were successfully transferred to the soil where they grew well for 7 to 10 weeks with 80% survivability (plate 11). So considering the survival rate it can be said that acclimatization potentiality of *Capsicum annum* is satisfactory.

Table 11. Survival rate of regenerated plantlets of *Capsicum annum*

Acclimatization	**No. of plants transplanted**	**No. of plants survived**	**Percentage of survival rate(%)**
In shade area with controlled atmosphere	20	16	80
In natural condition	16	12	75

a. In pot under shade condition

b. In natural condition

Plate 10: Acclimatization and establishment of plantlets a. In pot under shade condition b. In 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 March, 2018. The shoot tip of Pepper were used as experimental materials in the present investigation. The growth hormone BAP (1.0, 2.0, 3.0and 4.0 mg/L and IBA (1.0, 1.50, 2.00 and 2.50 mg/L) where used for *in vitro* regeneration. The experiments were arranged in Completely Randomized Design (CRD) with three replications.

BAP 3.0 mg/L gave the highest number of shoot (1.75, 2.75 and 3.50) at 14, 21 and 35 DAI and the control treatment found the lowest number of shoot (1.00, 1.50 and 2.15) at 14, 21 and 35 DAI. The highest number of leaves (3.50, 6.00 and 7.50) at 14, 21 and 35 DAI, respectively was noticed from the 3.0 mg/L BAP.

Maximum percentage (78%) of callus induction was noticed in treatment BAP 3.0 mg/L + IBA 2.0 mg/L and minimum percentage (37.%) was in treatment BAP 1.0 mg/L + IBA 1.0 mg/L. The maximum days (35.25) to shoot induction from callus was observed in control and the minimum duration (17.00 days) was obtained in BAP 3.0 mg/L + IBA 2.0 mg/L than rest of the result. The maximum percentage (75.00%) of shoot induction was observed in treatment BAP 3.0 mg/L + IBA 2.0 mg/L and the minimum percentage (32.15%) noticed in hormone free media.

The maximum number of shoot regenerated from callus (2.40, 2.23 and 3.76) was noticed from the BAP 3.0 mg/L + IBA 2.0 mg/L and the minimum number of shoot (0.56, 1.22 and 1.75 cm) at 14 DAI, 21 DAI and 35 DAI, respectively were noticed in treatment 1.0 mg/L + IBA 1.0 mg/L.

The highest percentage (82%) of root induction was induced in treatment 2.0 mg/L IBA and the lowest percentage (35%) was induced in hormone free media.

The highest number of root (3.25, 12.00 and 20.32) at 14, 21 and 35 DAI respectively was obtained in 2.0 mg/L IBA whereas the lowest number of root (1.10, 1.40 and 4.10) at 14, 21 and 35 DAI, respectively were noticed in control hormone free media.

From the above summary it is concluded that, the moderate dose of BAP 3.0 mg/L gave the best results for shoot regeneration in comparison with combine dose of BAP 3.0 mg/L with IBA 2.0 mg/L and IBA 2.0 mg/L gave the best performance. So, finally it can be concluded that, a convenient protocol of *in vitro* rapid regeneration of pepper (*Capsicum annum*) has been established which may contribute in 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 meristem, leaf and nodal segment can be used other than nodal segment for *in vitro* regeneration of pepper (*Capsicum annum* L.).

ii. Callus induction can be done with 2-4-D or other callus induction hormone for large number of shoot induction.

iii. Further study can be done with different concentrations and combinations of auxins and cytokinins group of hormones for rapid proliferation of (*Capsicum annuum)*.

iv. As micro-propagation techniques of pepper is not so well established in Bangladesh. So, further research on this crop should be continued because of its commercial use.

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APPENDICES

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

Source of variance	d.f	Days to shoot initiation	Percent (%) Shoot initiation	Number of Shoot		
				14 DAI	21 DAI	35 DAI
Treatment	3	45.234**	1332.225**	4.229**	8.167**	22.929**
Error	9	0.672	75.567	0.285	0.333	0.451
Total	12					

**= Significant at 1% level of Probability.

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

Source of variance	d.f	Number of leaves		
		14 DAI	21 DAI	35 DAI
Treatment	3	3.229**	8.916**	27.000**
Error	9	0.284	0.194	0.444
Total	12			

**= Significant at 1% level of Probability.

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

Source of variance	d.f	Length of shoot		
		14 DAI	21 DAI	35 DAI
Treatment	3	0.241**	0.501**	2.454**
Error	9	0.022	0.096	0.383
Total	12			

**= Significant at 1% level of Probability.

Appendix IV. Analysis of variance (ANOVA) of effect of different concentration ofBAP and IBA on Size of callus at different DAI

Source of variance	d.f	Size of callus		
		14 DAI	21 DAI	35 DAI
Treatment	9	1.241**	2.726**	5.741**
Error	30	0.082	0.124	0.085
Total	39			

**= Significant at 1% level of Probability

Appendix V. Analysis of variance (ANOVA) of effect of different concentration of BAP and IBA on days to callus initiation, percent of callus and number of shoot from callus at different DAI

Source of variance	d.f	Days to shoot initiation	Percent (%) Shoot initiation	Number of Shoot		
				14 DAI	21 DAI	35 DAI
Treatment	9	55.214**	1152.125**	3.249**	7.137**	20.949**
Error	30	0.572	68.367	0.483	0.543	0.621
Total	39					

**= 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		
		14 DAI	21 DAI	35 DAI
Treatment	3	7.869**	18.674**	24.334**
Error	9	0.164	0.462	0.221
Total	12			

**= Significant at 1% level of Probability.

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

Source of variance	d.f	Number of root		
		14 DAI	21 DAI	35 DAI
Treatment	3	2.750**	5.166**	15.895**
Error	9	0.138	1.000	3.784
Total	12			

**= Significant at 1% level of Probability.

Appendix VIII. Analysis of variance (ANOVA) of combined effect of different concentration of BAP 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		
				14 DAI	21 DAI	35 DAI
Treatment	9	56.280**	382.654**	0.631**	1.194**	1.483**
Error	30	o.463	152.679	0.568	0.681	0.973
Total	39					

**= Significant at 1% level of Probability.

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

Source of variance	d.f	Length of shoot		
		14 DAI	21 DAI	35 DAI
Treatment	9	0.292**	0.326**	0.274**
Error	30	0.304	0.413	1.092
Total	39			

**= Significant at 1% level of Probability.

Appendix X. Analysis of variance (ANOVA) of combined effect of differentconcentration of BAP and IBA on number of root at different DAI

Source of variance	d.f	number of root		
		14 DAI	21 DAI	28 DAI
Treatment	9	1.150**	2.194**	15.410**
Error	30	0.612	1.134	1.179
Total	39			

**= Significant at 1% level of Probability.

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

Source of variance	d.f	Length of root		
		14 DAI	21 DAI	28 DAI
Treatment	9	0.182**	0.945**	4.514**
Error	30	0.097	0.443	0.492
Total	39			

NS= Not Significant *= Significant at 5% level of Probability, **= Significant at 1% level of Probability