IN VITRO PROPAGATION OF BETEL VINE

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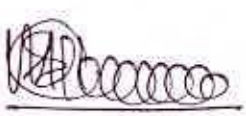

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CERTIFICATE

This is to certify that thesis entitled, **"*IN VITRO* PROPAGATION OF BETEL VINE"** *submitted to the Faculty of* **AGRICULTURE,** *Sher-e-Bangla Agricultural University, Dhaka, Bangladesh, in partial fulfillment of the requirements for the degree of* **MASTER OF SCIENCE** *in* **BIOTECHNOLOGY**, *embodies the result of a piece of bonafide research work carried out by* **MD. MAHABUB ELAHI**, *Registration No.* **09-03587** *under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.*

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

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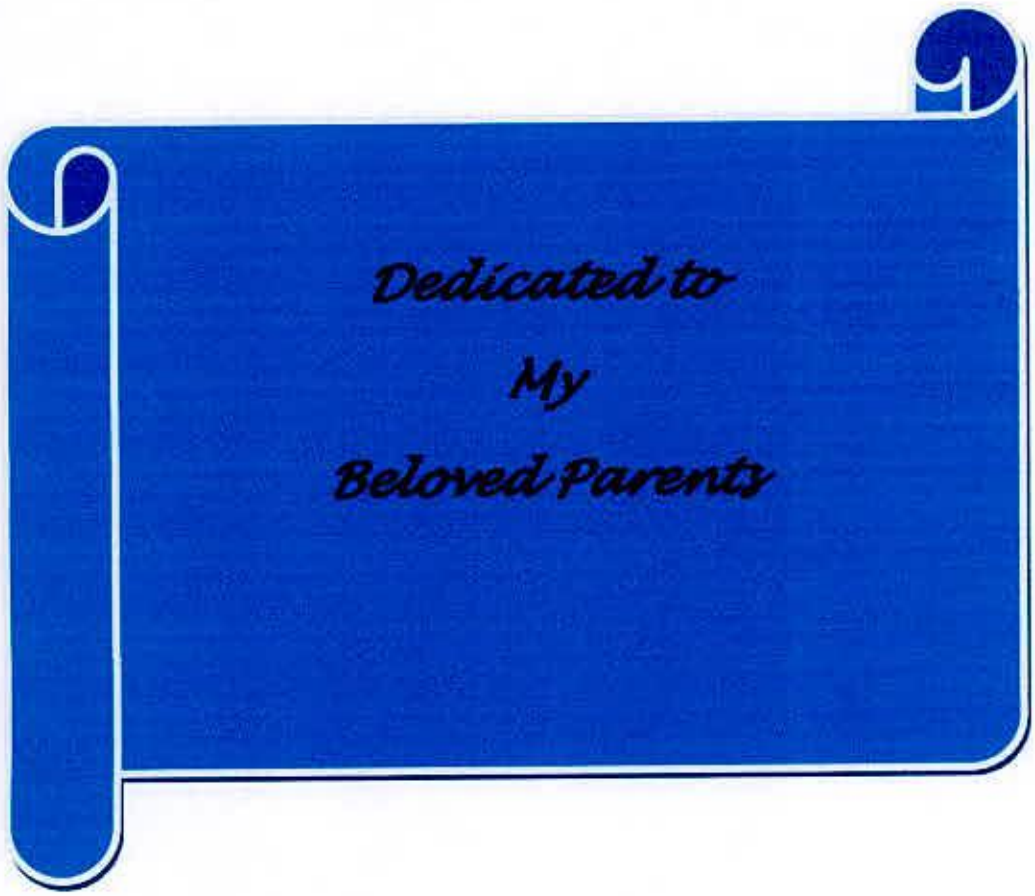
Dedicated to
My
Beloved Parents

ABBREVIATIONS AND ACRONYMS

Agril.	:	Agriculture
Biol.	:	Biological
cm	:	Centimeter
CRD	:	Completely Randomized Design
DMRT	:	Duncan's Multiple Range Test
Conc.	:	Concentration
DAI	:	Days After Inoculation
et al.	:	And others (at elli)
FAO	:	Food and Agricultural Organization
IASC	:	International Aloe Science Council
g/L	:	Gram per litre
BAP	:	6- Benzyl Amino Purine
BA	:	Benzyladenine
KIN	:	Kinetine
IAA	:	Indole acetic acid
IBA	:	Indole butyric acid
NAA	:	*a*- Napthalene acetic acid
2, 4-D	:	2,4- Dichlorophenoxy acetic acid
MS	:	Murashige and Skoog
Int.	:	International
J.	:	Journal
Mol.	:	Molecular
mg/L	:	Milligram per litre
µM	:	Micromole
PGRs	:	Plant Growth Regulators
Res.	:	Research
Sci.	:	Science
TDZ	:	Thidiazuron
CV	:	Co-efficient of Variation
^{o}C	:	Degree Celsius
etc.	:	Etcetera

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The Author

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BY

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ABSTRACT

The experiment was carried out on Betel vine (*Piper betle* L.) in Biotechnology Lab. of the Department of Biotechnology, Sher-e-Bangla Agricultural University, Sher-e-Bangla Nagar, Dhaka-1207 from the period of July 2014 to April 2015 to investigate the effect of BA, KIN, IAA and IBA on shoot proliferation, root formation and micropropagation protocol development. Nodal segment was used as explant. The highest percent (70%) of shoot proliferation and minimum days to shoot induction (21.67 days) were achieved on MS medium containing BA 1.0 mg/L + KIN 3.0 mg/L. Maximum average length of shoot (8.43 cm) was reported from media containing BA 0.5mg/L + KIN 4.0 mg/L. The highest percent (90%) of rooting and maximum 14.40 root/explant were reported in media treated with 3.0 mg/L IBA. Maximum 90% survival was observed in growth chamber and 83% during shade house and 64% in open atmosphere. A protocol for micropropagation of Betel vine. has been established and this experiment can be a useful tool for micropropagation of Betel vine.

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Introduction

CHAPTER I
INTRODUCTION

The leaves of Betel vine are commonly known as Pan in Bangladesh. It is an important cash crop in Bangladesh. It is also known as Nagaballi, Nagurvel, Saptaseera, Sompatra, Tamalapaku, Tambul, Tambuli, Vaksha Patra, Vettilai, Voojangalata etc in different parts of India (Guha and Jain, 1997). Most of the people in Bangladesh like chewing betel vine as habit, sometimes it is used as an item of rituals, etiquette and manners.

The Betel (*Piper betle* L.) is the leaf of a vine belonging to the Piperacae family, which includes pepper and Kava. The botanical name of betel vine is *Piper betle* L. It is a creeper with shiny, green heart-shaped leaves. The vine of Betel (*Piper betle* L) initiates many short adventitious roots (Hassan and Shahzad, 2005). The vine is a dioecious (male and female plants are different), shade loving perennial root climber. There are about 100 varieties of betel vine in the world, of which about 40 are found in India and 30 in West Bengal (Guha, 1997; Maity, 1989; Samanta, 1994). In Bangladesh most cultivated varieties of Betel vine are Desi Bangla, Bangla, Kali Bangla, Jhali, Sanchi, Bhabna, Mitha, Geso, Bonhoogly etc.

The most probable place of origin of Betel vine is Malaysia (Chattopadhyay and Maity, 1967). Inspite of its alienness, the plant is much more popular in India and Bangladesh than in any other country of the world since the antiquity. This would be evident from the numerous citations laid down in the ancient literature, particularly the Sub Continental scriptures. In these citations, importance of the leaves has been explained in relation to every sphere of human life including social, cultural, religious and even day-to-day life, which is very much relevant even these days. For example, a well-prepared betel quid is still regarded as an excellent mouth freshener and mild vitalizer, routinely served on the social, cultural and religious occasions like marriage, religious festivals. It is also used as a special item offered to the guests in order to show respect and for such traditional use of Betel leaf in our society, the leaf really stands alone without any parallel even today (Guha, 1997; Mehrotra, 1981).

In fact, this edible leaf has achieved an esteemed position in the human society right from the dawn of civilization, particularly in the countries like Bangladesh, Myanmar, China, India, Indonesia, Malaysia, Nepal, Pakistan, Philippines, South Africa, Sri Lanka, Thailand etc. (Jana, 1996; Khoshoo, 1981; Samanta, 1994; Sharma *et al.*, 1996), where leaves are traditionally used for chewing in their natural raw condition along with many other ingredients like sliced areca nut, slaked lime, coriander, aniseed, clove, cardamon, sweetener, coconut scrapings, ashes of diamond, pearl, gold and silver (Ayurvedic preparations), jelly, pepper mint, flavouring agent, fruit pulp etc. (CSIR, 1969).

Betel leaf is traditionally known to be useful for the treatment of various diseases like bad breath, boils and abscesses, conjunctivitis, constipation, headache, hysteria, itches, mastitis, mastoiditis, leucorrhoea, otorrhoea, ringworm, swelling of gum, rheumatism, abrasion, cuts and injuries etc as folk medicine while the root is known for its female contraceptive effects (Chopra *et al.*, 1956; Khanra, 1997). Further, the essential oil contained in the leaves possesses antibacterial, antiprotozoan and antifungal properties. Chakraborty and Shah 2011 studied on Antimicrobial, antioxidative and antihemolytic activity of Piper betel leaf extracts. Not only that, the betel leaves really does not have any match as a cheap, natural and easily available appetizer, digestive, mild stimulant, aphrodisiac and refreshing mastication. Chewing of betel leaves produce a sense of well-being, increased alertness, sweating, salivation, hot sensation and energetic feeling with exhilaration. It also increases the capacity to exercise physical and mental functions more efficiently for a longer duration but it may produce a kind of psychoactive effect causing a condition of mild addiction leading to habituation and withdrawal symptoms (Chu, 2001; Garg and Jain, 1996). Further, the leaves are very nutritive and contain substantial amount of vitamins and minerals and therefore, six leaves with a little bit of slaked lime is said to be comparable to about 300 ml of cow milk particularly for the vitamin and mineral nutrition. The leaves also contain the enzymes like diastase and catalase besides a significant amount of all the essential amino acids except lysine, histidine and arginine, which are found only in traces (Gopalan, 1984;Guha and Jain, 1997).

The betel leaves are also reported to possess antioxidant activity besides antimutagenic and anticarcinogenic properties particularly against the tobacco

carcinogens (Chang *et al.*, 2002b; Padma *et al.*, 1989a, Padma et al., 1989b; Wu *et al.*, 2004) due to the presence of ingredients like hydroxychavicol and chlorogenic acid (Amonkar *et al.*, 1989).

Contrary to the above, there are a few reports, which indicate that chewing betel leaves may independently produce carcinogenic effects (Chen *et al.*, 1999; Merchant *et al.*, 2000). Interestingly, it is also claimed that the inflorescence of betel vine contains carcinogens whereas the leaves possess anticarcinogenic agents. This practically indicates that parts of the same plant contain carcinogenic and anticarcinogenic substances (Wu *et al.*, 2004).

Bangladesh is the second largest grower of betel vine on about 14,175 hectare. Total annual production of the crop in Bangladesh is about 78,600 tons. The average yield is 2.47 tons per acre. But the acreage of Betel vine is decreasing fast because of some physical and socioeconomic barriers like unavailability of credit facilities, uncontrolled marketing system and infestation of diseases and pest and low quality planting materials (Islam 2005).

In spite of its enormous potentiality in both domestic and international market, the cultivation of Betel vine. in Bangladesh is not enough. Naturally Betel vine grows vegetatively and growth rate is very slow but its demand is high. The common propagation of Betel vine is conventional method by means of stem cutting which is inefficient to meet the demand of Betel vine leaves. Besides, vegetatively propagated plants often get systematically infected with a disease, the pathogens pass from one vegetative generation to next and ultimately entire population of a clonal variety may get infected with same pathogen and with latent viruses. As a result, the yield of betel vine decreases day by day.

Due to the huge utilization, the demand for quality planting material of Betel vine is increasing day-to-day. But current production of leaves is not enough for the fast growing demand of local and international market.

Hence, an alternative method is essential. *In vitro* technique offers a possibility to solve these problems. *In vitro* regeneration holds tremendous potential for the

production of high-quality planting materials as compare to conventional propagation. Micropropagation has the advantage of allowing rapid propagation in limited time and space. The problem of low productivity associated with conventional method can be minimized by using micropropagation techniques (Kalimuthu *et al.*, 2010).

Presence of plant growth regulators plays a significant role in a successful regeneration of any plant species. Among the plant growth regulators, cytokinin in culture medium is the most important factors for shoot proliferation (Liao *et al.*, 2004; Mamidala and Nanna, 2009; Hoque, 2010). Ali *et al.* (2005) reported, "the optimum combination of cytokinins and auxin critical to shoot regeneration".

So, mass propagation of uniform, healthy plants through tissue culture is the only viable technique for production of large numbers of betel vine in a short time. Though several attempts was taken for last few decades to develop tissue culture systems of *Piper spp.* but still the efficient regeneration protocols are requisite to develop a rapid, less expensive, efficient and easy method of micropropagation of Betel vine.

Hence, the present investigation, it has been carried out with the following objectives:

1. To establish *in vitro* propagation protocol of Betel vine.
2. To assess the combined effect of growth regulator for *in vitro* response.
3. To regenerate the plants that is genetically identical to the source material.
4. To acclimatize the propagated plantlets of Betel vine.

Chapter II
Review of literature

CHAPTER II
REVIEW OF LITERATURE

Tissue culture technique is the backbone of plant biotechnology. It is composed of micropropagation, somatic hybridization, somaclonal variation, regeneration of transgenic plant and cryopreservation. Plant tissue culture is a technique through which any plant part is used as an explant which cultured on a sterile nutrient medium in controlled temperature and light with the purpose of obtaining maximum number of plantlet. The pioneer work on culture of plant cells and tissues was started by Haberlandt (1902). He was the first person to culture isolated vegetative cells of higher plants in simple nutrient solutions. Embryos were first plant tissue to be successfully cultured *in vitro* on artificial media. Hanning (1904) successfully cultured embryos of Raphanus sativus, R. landra, R. caudatus and Cochlearia danica on Tollen's medium and obtained transplantable seedlings. White (1934) was the first to achieve success in continuously growing cultures of tomato root tips using sucrose, inorganic salt and yeast extract. A medium for rapid growth of tobacco tissue *in vitro* was developed by Murashige and Skoog (1962), popularly known as MS medium, which is one of the most widely used salt compositions for the purpose of plant regeneration. Nowadays, it is very common practice all over the world. But unfortunately, it is very limited in Bangladesh. However some related works already performed by different institutes. Some of the most relevant literature are cited below.

2.1. Explant

Zuraida *et al.* (2015) standardized a micropropagation protocol for a valuable medicinal plant, *Piper crocatum*. Shoot was initiated from internodes and apex shoots on MS medium supplemented with plant growth regulator. Three type of anti-browning, proline, PVP and charcoal were tested to reduce browning problem. Internodes showed better response for shoot induction than apex shoots. MS media containing 5 mg/L BAP, 0.5 mg/L 2, 4-D supplemented with charcoal was the most effective in shoot initiation, proliferation and showed less in browning. The developed complete plantlets with root were successfully acclimatized and established in glass house and the final survival rate was 70%.

Parida *et al.* (2011) studied on micropropagation of *Piper longum* through *in vitro* culture of nodal segment explants. Maximum number of shoots in *Piper longum* was obtained from nodal segment explants cultured on MS supplemented with 1mg/L benzyladenine and 1mg/L indoleacetic acid. Rooting was obtained from micro shoot cultured on MS supplemented with 1 mg/L benzyladenine and 0.2 mg/L indoleacetic acid.

Hussain *et al.* (2011) developed a protocol for micro-propagation of black pepper vine. The stem, leaf and shoot tip explants from mature vine were cultured on MS medium supplemented with different concentrations of plant growth regulators (2,4-D, BA, IBA). Best callus was produced on MS medium with 1.5 mg/l BA by shoot tip explant. Shoot regeneration was excellent on MS medium with 0.5 mg/l BA. The plantlets formed were rooted best on 1.5 mg/l IBA. The rooted plants were transplanted in soil medium and acclimatized in growth room.

Balbuena (2009) conducted an experiment to establish a cell suspension culture system for *Piper solmsianum*. Cell suspension culture obtained from petiole and leaf explants cultured in the presence of different plant growth regulator combinations (IAA, NAA, 2,4-D and BA). Root and indirect shoot adventitious formation, detected by histological analysis, was observed. Besides the different combinations of plant growth regulators, light regime and the supplement of activated charcoal (1.5 mg/l) were tested for callus induction and growth. Cultures maintained in light, on a 0.2 mg/l 2,4-D and 2 mg/l BA supplemented medium, and in the absence of activated charcoal, showed the highest calli fresh matter increment. From a callus culture, cell suspension cultures were established and their growth.

Bhat *et al.* (1992) established a protocol for plant regeneration from callus culture of *Piper longum* through organogenesis. Competent callus was initiated around the nodal ring using Murashige and Skoog (MS) medium supplemented with 1.0 mg/L alpha-napthaleneacetic acid and 0.2 mg/L N_6-benzyladenine. Optimum growth regulator concentration for shoot induction and shoot elongation were found to be 0.5 mg/L indole-3-acetic acid with 1.5 mg/L benzyladenine and 0.1 mg/L indole-3-acetic acid with 0.2 mg/L benzyladenine, respectively. Elongated shoots were rooted on half

strength MS having 0.1 mg/L indole-3-acetic acid. The rooted plants were successfully established in soil.

Datta *et al.* (2002) developed an efficient micropropagation protocol under an experiment named improved culture medium for micropropagation of *Piper longum* using shoot tip explants. Multiple shoots were induced from shoot tips cultured on agar-based Murashige and Skoog (MS) medium containing 4.44–22.19 M benzyladenine (BA) and 4.64–13.9 M kinetin (K). Maximum number of shoots were induced with 8.9 M BA and 4.64 M K. Adventitious shoot regeneration from leaf segments was achieved on MS containing 3.6–22.19 M BA along with 3.31–12.4 M picloram (P). Shoot differentiation occurred directly from the leaf bases without intermediale callus formation. Maximum shoot buds were obtained on MS medium with 17.76 M BA and 8.28 M P. Elongated shoots were separated and rooted in MS supplemented with 2.46 M indole butyric acid (IBA). Plantlets, thus developed were established in soil.

2.2. Plant growth regulator

Padhan (2015) carried out an experiment on micropropagation of *Piper longum* L. from nodal segment explants from one year old plants.The best shoot proliferation was observed in MS medium containing 1.0 mg/l Kinetin and 1.5 mg/l BA where 98 % of explants showed proliferation with highest rate of shoot multiplication (5-6 shoots per explant). Callus induction occurred in (1 mg/l) BA and (0.5 mg/l) Kinetin and 10-15 days of callus subculture initiation of greenish white shoot buds was observed. For rooting, the in vitro micro shoot were inoculated to MS basal media supplemented with 0.5 mg/l IAA and rooting was more profuse. The regenerated plantlets were successfully established in soil with survival rate 90%.

Domínguez *et al.* (2006) conducted an experiment on whole plant regeneration method from callus culture of *Piper auritum* through organogenesis derived from leaf tissue. Proliferating callus and shoot cultures derived from leaf tissue explants placed on Murashige and Skoog (MS) medium supplemented with 2.0 mg/L 2, 4-diclorophenoxyacetic acid (2,4-D) + 1.5 mg/L kinetin (KIN). Optimum combination of hormones for shoot induction was 0.5 mg/L 2,4-D and 1.5 mg/L kinetin (by volume) that resulted in a high shooting rate (49.6 shoots per explants). All of the

plant elongated when using a medium consisting of 0.1mg/L 2,4-D + 1 mg/L KIN. Elongated shoots were successfully rooted (100%) on half-strength MS medium supplemented with 2.0 mg/L indole-3-acetic acid. All plants survived to the growing conditions of a greenhouse.

Misra *et al.* (2004) studied over *in vitro* propagation of *Piper betle L.* Its growth was found well in different concentrations of BA (6-Benzylaminopurine), IBA (3-Indol butyric acid). . Maximum numbers of axillary shoots were obtained with 2 mg/L BA and 0.2 mg/L NAA as growth supplements. The plants were rooted in 0.25 mg/L IBA and hardened in the soil.

Khandekar *et al.* (2004) conducted an experiment on rapid multiplication of black pepper (*Piper nigrum*). On that experiment, the best initiation and multiplication of shoot obtained on MS medium with 0.5mg/l BAP + 0.05 mg/1 NAA. The regenerated shoots rooted best on medium containing 1 mg/L IBA with 0.5% activated charcoal.

Anand *et al.* (2000) worked on a rapid *in vitro* propagation protocol for *Piper barberi* Gamble (Piperaceae) through shoot tip and nodal explant cultures. Nodal explants with a single axillary meristem showed three times better response with respect to shoot proliferation when compared to shoot tip explants. The best shoot proliferation response of nodal explants was observed with a cytokinin combination of N_6-benzyladenine (4.43 μM) and kinetin (2.32 μM), with 88% bud break. The number of shoot initials (2.4) produced per nodal explant was twice the number of shoot initials (1.2) per shoot tip. An average of 6.9±0.58 adventitious shoots were observed from the proximal end of the internodal explants on Mursashige and Skoog (1962) (Ms) basal medium supplemented with N_6-benzyladenine (2.22 μM) and kinetin (0.46 μM). A multiplication rate of 82 shoots per explant could be achieved after 9 wk of subculturing. The *in vitro* shoots were rooted on one-half and one-quarter MS basal medium. The shoots rooted on one-quarter MS in the dark produced eight roots with an average root length of 3.36 cm and 98% survival. These plants were transferred to the field with a survival rate of 75%.

Bhat *et al.* (1995) carried out an experiment on plantlet regeneration from various explants of cultivated *Piper* species. On that experiment, morphogenetic potential of root, leaf, node and internode expiants of 3 cultivated *Piper* species 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 expiants 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. Shoot elongation and rooting were achieved in B_5 medium containing 0.5 μM benzyladenine and 1 μM indoleacetic acid, respectively. Regenerated plants were established in soil.

Chapter III
Materials and Methods

CHAPTER III

MATERIALS AND METHODS

3.1. Time and Location of the experiment

The present study was carried out during July, 2014 to April, 2015 at the Biotechnology Laboratory, Department of Biotechnology, Sher-e-Bangla Agricultural University, Sher-e Bangla Nagar, Dhaka-1207, Bangladesh. Materials and methods followed to conduct the present study have been presented in this chapter.

3.2. Experimental materials

3.2.1. Plant materials

Fresh, healthy and disease free Betel vine were used as experimental materials in the present study.

3.2.2. Source of materials

The planting materials of Betel vine were collected from farmers' field in Kalkini, Madaripur.

3.2.3. Types of explant

The healthy, disease free nodal part (1-1.5cm) of Betel vine was used as explants for the study.

3.2.4. Instruments and glassware

Metal instruments *viz.*, scalpels, forceps, spatulas, needless and aluminum foils were sterilized in an autoclave at a temperature of 121^0C for 40 minutes at 15 PSI pressure. The Borosil glassware was used for all the experiments. Culture bottles, pipettes , flat bottom flasks, beaker , petridishes, and measuring cylinders (25 ml, 50 ml, 100 ml, 500 ml and 1000 ml) were used for media preparation.

3.2.5. Culture media

Nutritional components and growth regulators play vital role on *in vitro* regeneration. MS (Murashige and Skoog, 1962) medium supplemented with different phytohormones as per treatments were used in culture medium for shoot induction, shoot multiplication, maintenance and induction of roots from multiplied shoots (Composition of MS media have been shown in appendix I). Hormones were added separately to different media according to the requirements. Stock solutions of hormones were prepared before of media preparation and stored at 4^0C temperature.

3.3. Stock solution of hormone

The first step in the preparation of the medium were the preparation of hormone stock solutions. Separate stock solution of hormones was prepared by dissolving the desired quantity of ingredients to the appropriate solvent and made the final volume with distilled water and stored in a refrigerator at 4^0C for later use.

The following growth regulators and concentrations were used in this present investigation.

Auxins

Indole butyric acid (IBA) 1.0, 1.5, 2.0 and 3.0 mg/L

Indole acetic acid (IAA) 1.0, 1.5, 2.0 and 3.0 mg/L

Cytokinins

Benzyladenine (BA) 0.5, 1.0, 1.5 and 2.0 mg/L

Kinetin (KIN) 1.0, 2.0, 3.0 and 4.0 mg/L

These hormonal supplements were dissolved in proper solvent as shown against each of them.

Hormones (Solute)	Solvent
BA	1 N NaOH
Kin	1 N NaOH
IBA	99.99% ethyl alcohol
IAA	99.99% ethyl alcohol

To prepare the stock solution of hormones (1mg/ml), 100 mg of solid hormone was placed in a small beaker and then dissolved with 99.99% ethyl alcohol and 1N NaOH. Finally the volume was made upto 100 ml by the addition of sterile distilled water using a measuring cylinder. The prepared hormone solution was then labeled and stored at 4 ± 1^0C for use upto two month. (MS media was purchased, Duchefa Biochemie, Netherland and growth regulators were purchased from Sigma, USA.

3.4. The preparation of MS media

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

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

Step-2. 5 g of ready MS and 30 g of sucrose was added and gently stirrered to dissolve these ingredients completely with the help of a hot plate magnetic stirrer.

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

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

Step-5. The pH was adjusted at 5.8.

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

3.5. Sterilization

For *in vitro* techniques, aseptic condition is a prerequisite. So, all instruments, glassware and culture media were sterilized.

3.5.1. Sterilization of culture medium

The culture vessels containing the medium were autoclaved with 1.06 kg/cm^2 (15 PSI) of pressure at 121^{0}C for 20 minutes. After autoclaving the culture vessels (vials) containing the medium were allowed to cool in culture racks. After autoclaving the media were stored in at 25±2 °C for several hours to make it ready for inoculation with explants.

3.5.2. Sterilization of glassware and instruments

Beakers, test tubes, conical flasks, pipettes, metal instruments *viz*., forceps, scalpels, needless, spatulas and aluminum foils were sterilized in an autoclave at a temperature of 121^{0}C for 35 minutes at 1.06 kg/cm^2 (15 PSI) pressure.

3.5.3. Sterilization of culture room and transfer area

The culture room was initially cleaned by gently washing all over the floors and walls with detergent or Lysol (germicide) followed by wiping with 70% ethyl alcohol. The process of sterilization was repeated at regular intervals. Generally, switching on the laminar airflow cabinet and sterilized the cabinet by wiping the working surface with 70% ethyl alcohol and then UV light was on for 30 minutes so that the working area

of the cabinet is sterilized. After sterilization the cabinet was delayed for at least 5 minutes to ensure safe environment.

3.6. Precaution to ensure aseptic condition

The cabinet was usually started half an hour before use and wiped with 70% ethyl alcohol to reduce the chances of contamination. The instruments like forceps, scalpels, needles etc. were pre-sterilized by autoclaving and subsequent sterilization was done by dipping in 70% ethyl alcohol followed by flaming and cooling. Hands were also sterilized by wiping with 70% ethyl alcohol. Aseptic conditions were followed during each and every operation to avoid the contamination of culture.

3.7. Explants preparation and culture

3.7.1.1. Preparation of explants

The nodal segment was the explant. It was obtained from fresh, healthy and disease free Betel vine grown under field conditions and was brought to the preparation room. The vine was washed thoroughly under running tap water. The roots and outer tissues of the vine were removed with the help of a sharp knife.

3.7.1.2. Surface sterilization of explants

The nodal segment of 2 to 3 cm size was taken in a beaker (Plate 1). Surface sterilization of explants was done as follows:

i. The nodal segments were cut as small size (2 to 3 cm) and rinsed with running tap water.
ii. The nodal segments were treated with 70% ethanol for 5 minutes.
iii. Washing with distilled water was done for several times.
iv. The explants were immersed in 0.1% $HgCl_2$ within a beaker and added 3-4 drops of Tween-20 for about 4-5 minutes with constant shaking in clockwise and anticlockwise direction.
v. The explants were rinsed with sterilized distilled water for at least 4 times to make the material free from chemical and ready for inoculation in culture media.
vi. Finally the explants were transferred to the MS media carefully.

Plate 1. Explants for placement in culture media.

3.7.2. Culture of explant

3.7.2.1. Inoculation of culture

The sterilized explants were inoculated carefully following proper sterilization process within laminar airflow cabinet. Prior to use, the surface of the laminar flow bench was swabbed down with 70 % ethyl alcohol and the interior sprayed with the same alcohol. All glassware, instruments and media were steam-sterilized in an autoclave. During the course of the work, necessary instruments were placed in a beaker containing 70 % ethanol and were flamed repeatedly using a spirit burner. The worker's hands and forearms were washed thoroughly with soap and water and repeatedly sprayed with 70% alcohol during working period. The mouth of all culture vial was flamed before and after positioning of the explant on the medium.

For inoculation, explants were transferred to large sterile glass petridish or glass plate with the help of sterile forceps under strict aseptic conditions. Here the explants were further trimmed and extra outer leaves were removed. After cutting the explants into suitable size (1.5-2 cm), explants were inoculated in MS medium supplemented with plant growth regulators (Plate 2). After inoculation the mouth of bottle was quickly flamed and capped tightly. After proper labeling, mentioning media code, date of inoculation etc. the bottles was transferred to growth room.

Plate 2. Inoculation of explant

3.7.2.2. Incubation

The culture vials transferred to culture racks and allowed to grow in controlled environment. The temperature of the growth room was maintained within 25±1° C by an air conditioner and 8/16 hour photo period was maintained along with light intensity of 2000-3000 lux for proper growth and development of culture.

3.7.2.3. Maintenance of proliferating shoots

The explants were cultured on MS nutrient medium supplemented with different concentration of BA alone or in combination of KIN. Percentage of explants showing shoot proliferation, days for shoot induction, number shoots per explants, average length of shoots, and number of leaves per explant were considered as parameter for evaluating this experiment. After successful shoot proliferation, subculture was done with newly form shoots. Shoots ware excised in aseptic condition with help of sterile scalpel blade and sterile forceps and transferred to new MS media which was supplemented with same concentration of growth hormones in order to increase budding frequency. The observations on development pattern of shoots were made throughout the entire culture period. Data recording was started after 3 weeks from inoculation.

3.7.2.4. Regeneration of plants from *in vitro* proliferated buds

In vitro proliferated micro shoots were separated and each of the micro shoot was placed on culture medium, which was supplemented with particular concentration of hormone for shoot differentiation.

3.7.2.5. Root induction of regenerated shoots

Newly formed shoots with adequate length were excised individually from the culture vial and transferred to rooting media. Two types of auxin (IAA and IBA) were used separately in different concentration (1.0, 1.5, 2.0and 3.0 mg/L) along with MS media. The observations on development pattern of roots were made throughout the entire culture period. Data were recorded from 3rd week of inoculation.

3.8. Acclimatization of the regenerated plantlets

Regenerated plantlets were transplanted to pots (10×15cm) containing soil and cow dung in 1:1 ratio and soil mixture were treated with a solution of 1% IBA. Occasional spray of water was done to prevent sudden desiccations and maintain high humidity around the plantlets. Initially the plantlets were hardened in growth chamber. Then after 2 weeks, exposed to lower humidity and higher light intensity. Finally, after 20 days plantlets were transferred to natural environment.

3.9. Treatments

Four experiments were conducted to assess the effect of different concentrations of BA and KIN on shoot proliferation and IBA and IAA on subsequent rooting of the multiplied shoot.

Sub-experiment 1. Effect of BA on shoot induction potentiality in Betel vine.

In this experiment, nodal segment of Betel vine were used as sources of explant to investigate the effect of BA.

Treatments: Four levels of BA (0.5, 1.0, 1.5 and 2.0 mg/L) & and a control (0.0 mg/L) were used. The treratments were arranged in Completely Randomized Design (CRD) with 3 replications. Each replication contained 10 culture vials.

Sub-experiment 2. Effect of BA and KIN on shoot induction potentiality in Betel vine.

In this sub-experiment 16 combinations of BA and KIN were practiced. The combinations were as follows

T_2= BA 0.50 mg/L+1.00 mg/L KIN

T_3 =BA 1.00 mg/L+1.00 mg/L KIN

T_4 = BA 1.50 mg/L+1.00 mg/L KIN

T_5 = BA 2.00 mg/L+1.00 mg/L KIN

T_6 = BA 0.50mg/L +2.00 mg/L KIN

T_7 = BA 1.00 mg/L+2.00 mg/L KIN

T_8 = BA 1.50 mg/L+2.00 mg/L KIN

T_9 = BA 2.00 mg/L+2.00 mg/L KIN

T_{10} =BA 0.50 mg/L+3.00 mg/L KIN

T_{11}= BA 1.00 mg/L+3.00 mg/L KIN

T_{12} = BA 1.50mg/L+3.00 mg/L KIN

T_{13} = BA 2.00 mg/L+3.00 mg/L KIN

T_{14} = BA 0.50 mg/L+4.00mg/L KIN

T_{15} = BA 1.00 mg/L+4.00 mg/L KIN

T_{16} = BA 1.50 mg/L+4.00 mg/L KIN

T_{17} = BA 2.00 mg/L+4.00 mg/L KIN

And T_1 = control (0.0 mg/L)

The experiments were arranged in Completely Randomized Design (CRD) with 3 replications. Ten culture vials were considered as single replication.

Sub-experiment 3. Effect of IAA and IBA on root induction potentiality micro propagated shoots in Betel vine.

Four levels of IAA and IBA (1.0, 1.5, 2.0 and 3.0 mg/L) and control (0.0 mg/L) were used as treatments. The treatments were arranged in Completely Randomized Design (CRD) with 3 replications. Ten culture vials were considered as single replication.

Sub-experiment 4. *Ex vitro* acclimatization and establishment of plantlets on soil.

Tissue culture derived plantlets were acclimatized in growth chamber, shade house and open atmosphere to find out the survival percentage.

3.10. Data collection

Data were collected on the effect of different treatments on shoot parameters recorded at 3, 5, and 8 weeks after induction (WAI) and root proliferation on different treatments recorded at 3, 5 and 8 weeks after induction (WAI). The following observations were recorded in cases of shoot and root formation under *in vitro* condition.

1. Percent of explants showing shoot induction
2. Days to shoot induction
3. No. of shoot per explant
4. Average length of shoot (cm)
5. No. of leaf per explant
6. Percent of explants showing root induction
7. Days to root induction
8. No. of root per explant
9. Average length of root (cm)

3.10.1. Regeneration potentiality (%)

Regeneration potentiality was calculated by percentage of explants responded using the following formula.

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

3.10.2. Days to shoot induction

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

3.10.3. Number of shoot per explant

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

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

3.10.4. Shoot length (cm)

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

3.10.5. Number of leaf

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

3.10.6. Percent of explants showing root induction

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

3.10.7. Number of root

The number of root per plantlet was counted and the mean was calculated.

3.10.8. Length of root (cm)

Root length was measured in centimeter from the base to the tip of the roots and the mean was calculated.

3.10.9. Percentage of established plantlets

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

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

3.10.10. Calculation of percent increase over control (%)

The percent increase over control was calculated by using the following formula:

$$\text{Percent increase over control} = \frac{X1 - X2}{X2} \times 100$$

Where, X_1 = the mean of treated explants,

X_2 = the mean of untreated explants

3.11. Statistical analysis of data

The experiment was laid out as completely randomized design (CRD) with three replications per treatment. Data were statistically analyzed by analysis of variance (ANOVA) technique and differences among treatment means were compared by using Duncan's multiple range test (DMRT) at 5% probability level using MSTAT-C (1990) program.

Chapter IV
Results and Discussion

CHAPTER IV

RESULTS AND DISCUSSION

Four separates experiments were conducted to investigate the effect of different growth hormones in shoot proliferation and root induction in Betel vine. The overall objective of the present study was to develop a system for the mass propagation of Betel vine. These experiments were conducted at the Biotechnology Lab, Department of Biotechnology, Sher-e-Bangla Agricultural University. The results of the experiments are presented and discussed in this chapter with Plates (1-10), Figures (1-12), Tables (1-9). Analyses of variance in respect of all the parameters have been presented in Appendices I- XXVII.

4.1. Sub-experiment 1. Effect of BA on shoot induction potentiality in Betel vine.

The result of the effect of different concentrations of BA has been presented under following headings with Figure (1-4) and Table (1).

4.1.1. Days to shoot induction

Significant variations were observed among different concentrations of BA on days to shoot induction. The maximum days to shoot induction were recorded in control (38.67 days) treatment where minimum 25 days was needed in 1.0 mg/l BA (Figure 1).

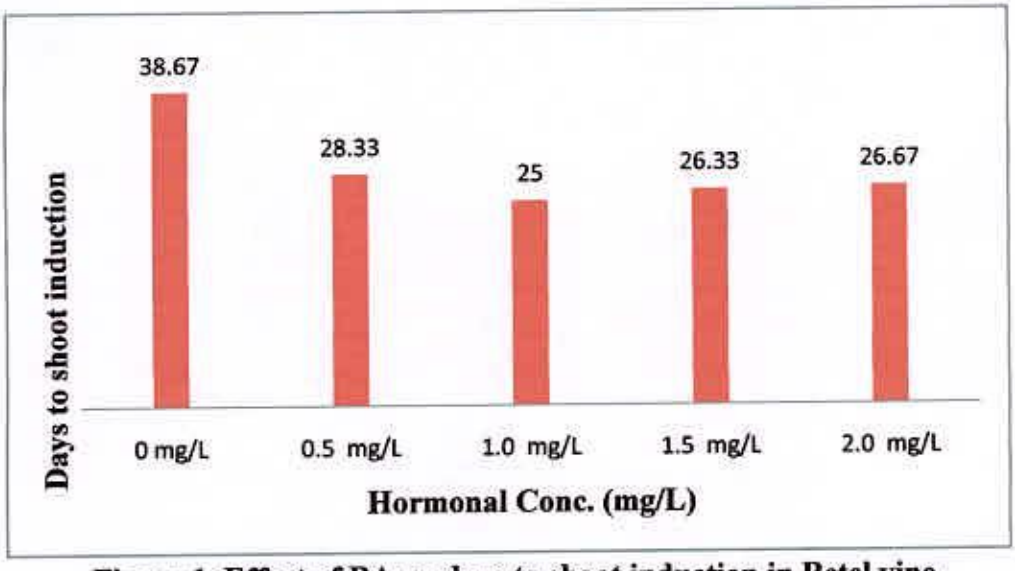

Figure 1. Effect of BA on days to shoot induction in Betel vine.

4.1.2. Percent of explants showing shoot induction

There was significant variation on percent of explants showing shoot induction at different concentrations of BA. Maximum shoot induction (55%) was found in treatment 1.0 mg/L BA and minimum percentage (25%) was induced in hormone free media (Figure 2). Hussain *et al.* (2011) also observed the influence of BA on shoot regeneration.

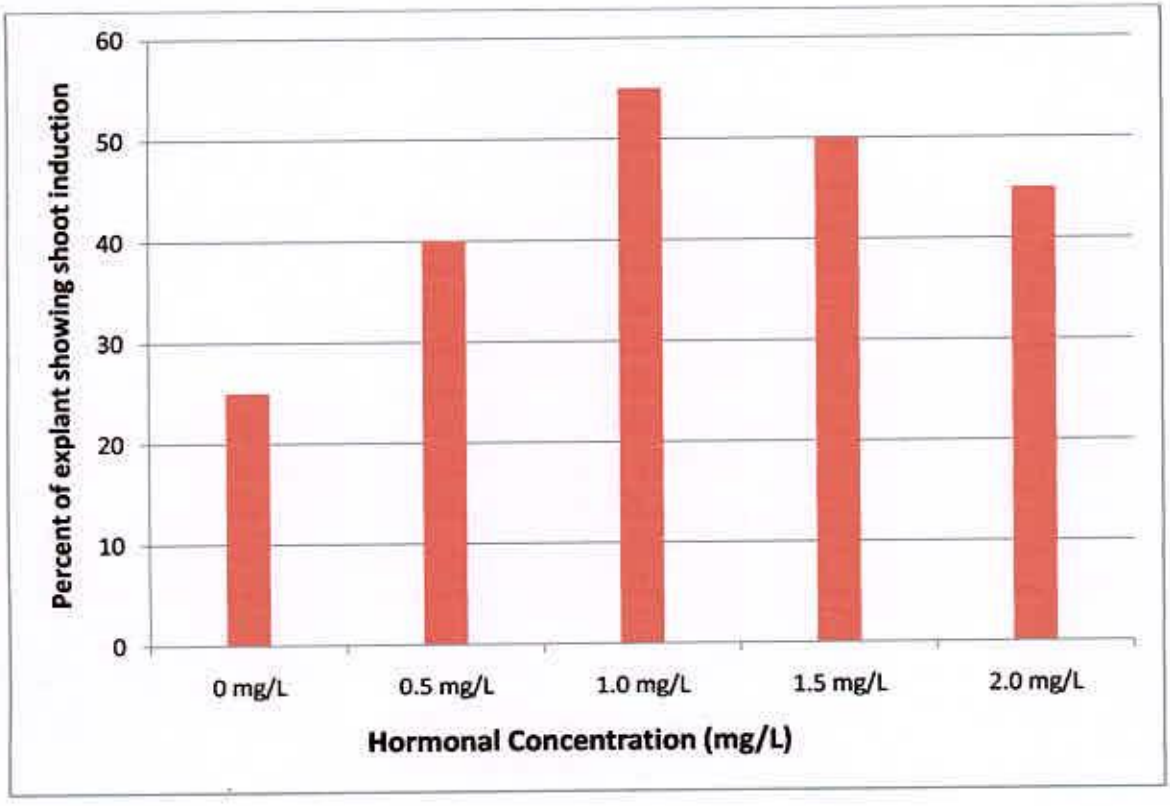

Figure 2. Effect of BA on percent of explants showing shoot induction in Betel vine.

4.1.3. Number of shoot per explant

There was significant influence of different concentrations of BA on the number of shoots per explant. Data were recorded after 3, 5 and 8 weeks of inoculation on MS media. The results have been presented in Figure 3. There was no significant variation at 3WAI among different concentration of BA. 2.0 mg/L BA gave the highest

number of shoots 1.46 and 2 at 5WAI and 8WAI respectively whereas the lowest number of shoots 1.13 and 1.26 at 5WAI and 8WAI respectively was found with hormone free media (Figure 3). Hussain *et al.* (2011) observed that shoot regeneration was excellent on MS media supplemented with 0.5 mg/L BA in black peper.

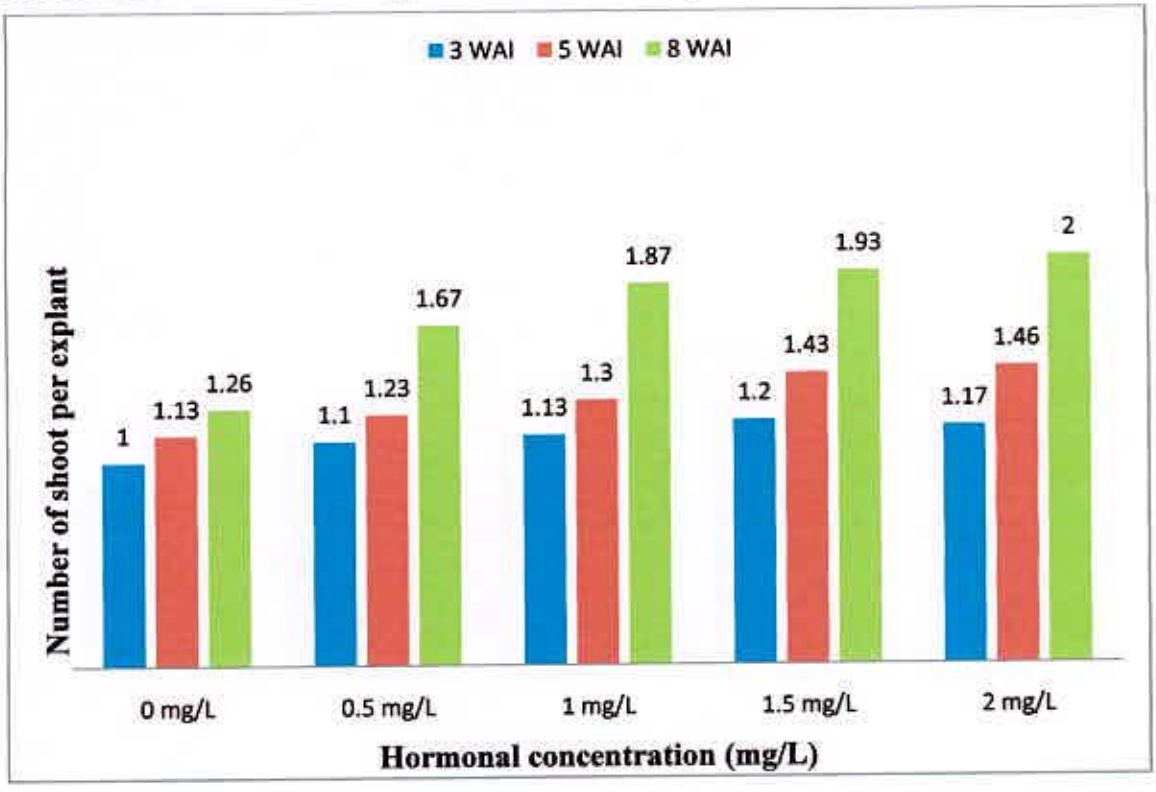

Figure 3. Effect of BA on the number of shoot per explant in Betel vine.

4.1.4. Average length of shoot (cm)

The results of average length of shoot have been presented in Table 1. Data were recorded after 3, 5 and 8 weeks of inoculation on MS media. There was significant influence of different concentrations of BA on the number of shoot per explant. The maximum average length of shoot 2.36 cm, 2.93 cm and 3.85 cm at 3WAI, 5WAI and 8WAI respectively was noticed from the 1.5 mg/L BA which was statistically different from rest of others. Whereas the minimum length 1.6 cm, 2.06cm, 2.16 cm at 3WAI, 5WAI and 8WAI respectively were noticed in control treatment (Table 1). Considering the percent increase of average length of shoot over control, 1.5 mg/L

showed the highest increase 178.24% at 8WAI. The minimum increase over control (129.62%) was observed in 1.0 mg/L at 8WAI.

Table 1. Effect of BA on average length of shoot (cm) in Betel vine.

Treatments	Name of the phytohormone	Phytohormone concentration (mg/L)	Average length of shoot			
			Length of shoot (cm) (3WAI)	Length of shoot (cm) (5WAI)	Length of shoot (cm) (8WAI)	% increase over control
T_1	Control	0	1.60c	2.06c	2.16c	-
T_2	BA	0.5	1.67bc	2.56ab	2.87b	132.87
T_3	BA	1.0	1.73bc	2.53b	2.80b	129.62
T_4	BA	1.5	2.36a	2.93a	3.85a	178.24
T_5	BA	2.0	1.76b	2.43b	2.90b	134.25
$LSD_{0.05}$			3.87	3.32	2.81	-
CV %			0.1286	0.1522	0.1409	-

4.1.5. Number of leaf per explant

Significant influence was found with different concentrations of BA treatment on the number of leaf. The maximum 3.33 leaves were recorded with 2.0 mg/L BA and the minimum 2.1 in case of control treatment (Figure 4).

Plate 3. Effect of 2.0 mg/L BA on number of leaves per explant in Betel vine at 8 WAI.

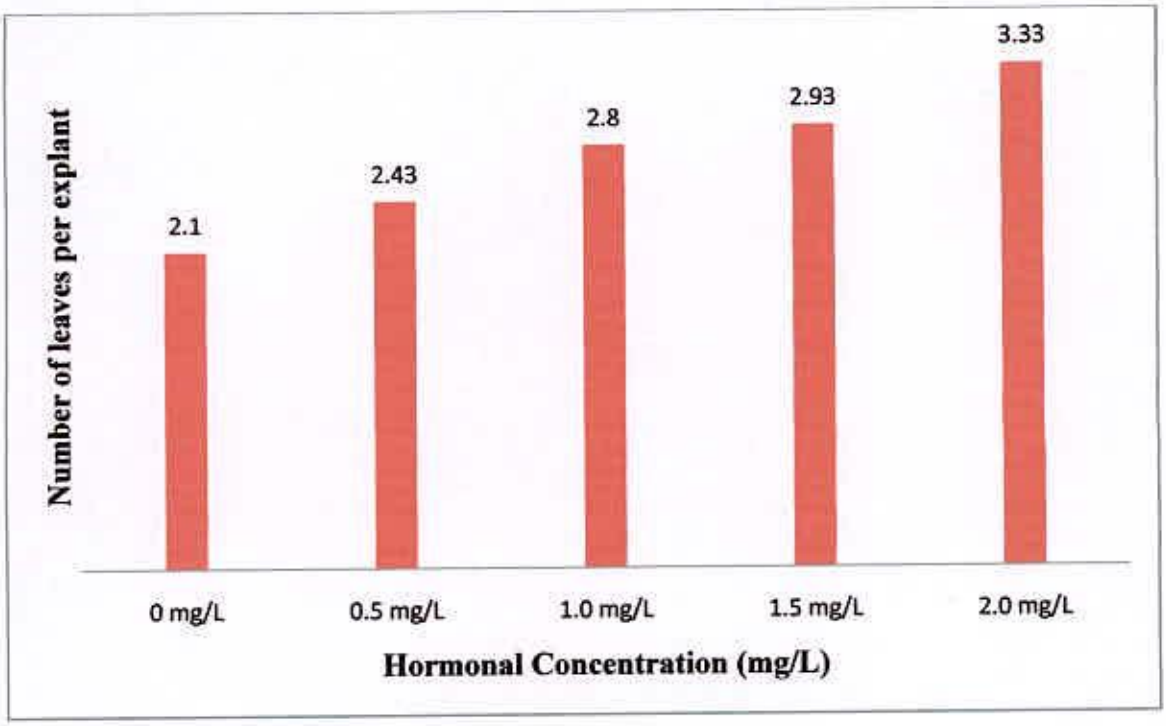

Figure 4. Effect of BA on the number of leaves in Betel vine.

4.2. Sub-experiment 2. Combined effect of BA and KIN on shoot induction potentiality in Betel vine.

The results of the combined effect of different concentrations of BA + KIN have been presented under following headings with Table 2-5 and Plate 3-6.

4.2.1. Percent of explants showing shoot induction

Significant variation was observed for the combine use of BA+KIN on the percent of shoot induction explants. Maximum percentage (70%) of shoot induction was noticed in treatment 1.5 mg/L BA + 3.0 mg/L KIN and minimum percentage (25%) was induced in hormone free media (Table 2). Anand *et al.* (2000) observed 85% bud break obtain in MS supplemented with combination of N_6-benzyladenine (4.43 μM) and kinetin (2.32 μM). Padhan (2015) reported that 98% of explants showed shoot proliferation on MS supplemented with 1.0 mg/l Kinetin and 1.5 mg/l BA.

Table 2. Combined effect of BA and KIN on percent of explants showing shoot induction in Betel vine.

Treatments	Name of the Phytohormones	Phytohormones concentration (mg/L)	Number of explant inoculated	Number of explant initiated shoot	Percent(%) of explants showing shoot induction
T_1	Control	0.0	20	5	25
T_2	BA+KIN	0.50+1.00	20	7	35
T_3	BA+KIN	1.00+1.00	20	8	40
T_4	BA+KIN	1.50+1.00	20	10	50
T_5	BA+KIN	2.00+1.00	20	9	45
T_6	BA+KIN	0.50+2.00	20	11	55
T_7	BA+KIN	1.00+2.00	20	9	45
T_8	BA+KIN	1.50+2.00	20	10	50
T_9	BA+KIN	2.00+2.00	20	10	50
T_{10}	BA+KIN	0.50+3.00	20	11	55
T_{11}	BA+KIN	1.00+3.00	20	12	60
T_{12}	BA+KIN	1.50+3.00	20	14	**70**
T_{13}	BA+KIN	2.00+3.00	20	11	55
T_{14}	BA+KIN	0.50+4.00	20	12	60
T_{15}	BA+KIN	1.00+4.00	20	11	55
T_{16}	BA+KIN	1.50+4.00	20	9	45
T_{17}	BA+KIN	2.00+4.00	20	10	50

4.2.2. Days to shoot induction

Variations were observed among different concentrations of BA+KIN on days to shoot induction. The maximum days to shoot induction was recorded in control (38.67 days) and 1.0 mg/L BA+3.0 mg/L KIN required minimum (21.67 days) (Table 3).

Plate 4. Initiation of shoot induction at 21.67 DAI in Betel vine when MS supplemented with 1.0 mg/L BA+3.0 mg/L KIN.

Table 3. Combined effect of BA and KIN on shoot induction potentiality

Treatments	Name of the Phytohormones	Phytohormones concentration (mg/L)	Shoot induction potentiality				
			Days to shoot induction	No. of shoot per explant (3 WAI)	No. of shoot per explant (5 WAI)	No. of shoot per explant (8 WAI)	% increase over control (8 WAI)
T_1	Control	0.0	38.67a	1.00i	1.13i	1.26i	-
T_2	BA+KIN	0.50+1.00	25.00bc	1.03hi	2.13h	2.76h	219.04
T_3	BA+KIN	1.00+1.00	25.33b	1.13gh	2.23gh	2.86h	226.98
T_4	BA+KIN	1.50+1.00	24.33bcd	1.23fg	2.36fg	3.03g	240.47
T_5	BA+KIN	2.00+1.00	25.00bc	1.23fg	2.40efg	3.10fg	246.03
T_6	BA+KIN	0.50+2.00	22.67de	1.33ef	2.56de	3.16fg	250.79
T_7	BA+KIN	1.00+2.00	23.00de	1.43de	2.56de	3.23f	256.34
T_8	BA+KIN	1.50+2.00	23.67bcd	1.40de	2.50def	3.23f	256.34
T_9	BA+KIN	2.00+2.00	23.00de	1.47d	2.63cd	3.46e	274.03
T_{10}	BA+KIN	0.50+3.00	22.67de	1.50d	2.76bc	3.90c	309.52
T_{11}	BA+KIN	1.00+3.00	21.67e	2.03a	3.46a	4.63a	367.46
T_{12}	BA+KIN	1.50+3.00	23.00de	1.86b	2.93b	4.13b	327.77
T_{13}	BA+KIN	2.00+3.00	23.67bcd	1.83b	2.86b	3.96c	314.28
T_{14}	BA+KIN	0.50+4.00	23.33cde	1.83b	2.76bc	3.83c	303.96
T_{15}	BA+KIN	1.00+4.00	24.00bcd	1.63c	2.60cd	3.86c	306.34
T_{16}	BA+KIN	1.50+4.00	24.00bcd	1.80b	2.76bc	3.66d	290.47
T_{17}	BA+KIN	2.00+4.00	24.00bcd	1.66c	2.66cd	3.63d	288.09
CV%		-	3.83	4.68	3.88	2.26	-
LSD value		-	1.558	0.1173	0.1659	0.1285	-

*WAI=Weeks After Inoculation. In a column, mean values with the same letters are not statistically different from each other at 5% probability by DMRT

4.2.3. Number of shoot per explant

Data were recorded after 3, 5 and 8 weeks of inoculation on MS media. There was significant influence of different concentrations of BA+KIN on the number of shoot per explant after 3rd weeks of inoculation. The results have been presented in Table 3. The treatment 1.0 mg/L BA+3.0 mg/L KIN gave the highest number of shoot 2.03, 3.46 and 4.63 at 3WAI, 5WAI and 8WAI respectively whereas the lowest number of shoot 1.0, 1.13 and 1.26 at 3WAI, 5WAI and 8WAI respectively was found with hormone free media (Table 3). Considering the percent increase of number of shoot per explant over control, 1.0 mg/L BA+3.0 mg/L KIN showed the highest increase 367.46% at 8WAI. The minimum increase over control (219.04%) was observed in 0.5 mg/L BA+1 mg/L KIN at 8WAI. Padhan (2015) reported that 5-6 shoots per explants obtain in MS containing combination of 1.0 mg/l Kinetin and 1.5 mg/l BA. Anand *et al.* (2000) observed 6.9 shoots per explants on MS supplemented with combination of with N_6-benzyladenine (2.22 μM) and kinetin (0.46 μM).

Plate 5. Combined effect of 1.0 mg/L BA+3.0 mg/L KIN on the number of shoot per explant in Betel vine at 8WAI.

4.2.4. Number of leaf per explant

The Number of leaf per explant was significantly different according to the various concentrations of BA+KIN supplemented. The results have been presented in Table 4. The maximum number of leaf per explant (4.8) was noticed from 1.0 mg/L BA+3.0 mg/L KIN and statistically different from rest of others, whereas the minimum 2.1 in control. Considering the percent increase of number of leaf per explant over control,

1.0 mg/L BA+3.0 mg/L KIN showed the highest increase 190.24% at 8WAI. The minimum increase over control (152.21%) was observed in 1.0 mg/L BA+1 mg/L KIN at 8WAI.

Table 4. Combined effect of BA and KIN on the number of leaf in Betel vine.

Treatments	Name of the Phytohormones	Phytohormones concentration (mg/L)	No. of leaf per explants at 8WAI	% increase over control at 8WAI
T_1	Control	0.0	2.1h	-
T_2	BA+KIN	0.50+1.00	3.3g	153.65
T_3	BA+KIN	1.00+1.00	3.2g	152.21
T_4	BA+KIN	1.50+1.00	3.6f	160.97
T_5	BA+KIN	2.00+1.00	3.5f	158.53
T_6	BA+KIN	0.50+2.00	3.6f	160.97
T_7	BA+KIN	1.00+2.00	3.7ef	163.41
T_8	BA+KIN	1.50+2.00	4.0cd	170.73
T_9	BA+KIN	2.00+2.00	4.1bc	173.17
T_{10}	BA+KIN	0.50+3.00	4.3b	178.04
T_{11}	BA+KIN	1.00+3.00	4.8a	190.24
T_{12}	BA+KIN	1.50+3.00	4.2b	175.60
T_{13}	BA+KIN	2.00+3.00	4.2b	175.60
T_{14}	BA+KIN	0.50+4.00	4.2b	175.60
T_{15}	BA+KIN	1.00+4.00	3.9de	168.29
T_{16}	BA+KIN	1.50+4.00	3.7ef	163.41
T_{17}	BA+KIN	2.00+4.00	3.7ef	163.41
CV%		-	1.66	-
LSD value		-	0.1892	-

Values in the column are the means of five replicates. In a column, mean values with the same letters are not statistically different from each other at 5% probability by DMRT.

4.2.5. Average length of shoot (cm)

With different concentrations of BAP+KIN, significant influence was found on the average length of shoot. The results of average length of shoot have been presented in Table 5. The maximum average length of shoot (3.93 cm, 4.86 cm and 8.43 cm at 3WAI, 5WAI and 8WAI) was noticed from the 0.5 mg/L BA + 4 mg/L KIN which were statistically different from rest of others (Plate 6). It was the minimum 1.6 cm, 2.06 cm, and 2.16 cm at 3WAI, 5WAI and 8WAI in control (Table 5). Considering the percent increase of average length of shoot over control, 0.5 mg/L BA+4 mg/L KIN showed the highest increase (390.27%) at 8WAI. The minimum increase over control (171.29%) was observed in 0.5 mg/L BA+ 1.0 mg/L KIN at 8WAI.

Plate 6. Combined effect of 0.5 mg/L BA+4.0 mg/L KIN on the length of shoot per explant in Betel vine at 8WAI.

Table 5. Combined effect of BA and KIN on length of shoot in Betel vine.

Treatments	Name of the Phytohormones	Phytohormones concentration (mg/L)	Length of shoot			
			Length of shoot (cm) 3WAI	Length of shoot (cm) 5WAI	Length of shoot (cm) 8WAI	% increase over control 8WAI
T_1	Control	0.0	1.60k	2.06l	2.16n	-
T_2	BA+KIN	0.50+1.00	2.30j	3.13k	3.70m	171.29
T_3	BA+KIN	1.00+1.00	2.56i	3.46j	3.90lm	180.55
T_4	BA+KIN	1.50+1.00	2.76h	3.76i	4.16jk	192.59
T_5	BA+KIN	2.00+1.00	3.00g	3.83i	4.10kl	189.81
T_6	BA+KIN	0.50+2.00	3.23f	4.03h	4.20jk	194.44
T_7	BA+KIN	1.00+2.00	3.43de	4.03h	4.36j	201.85
T_8	BA+KIN	1.50+2.00	3.53cd	4.10h	4.76hi	220.37
T_9	BA+KIN	2.00+2.00	3.43de	4.13gh	4.60i	212.96
T_{10}	BA+KIN	0.50+3.00	3.46de	4.23fg	4.93h	228.24
T_{11}	BA+KIN	1.00+3.00	3.43de	4.33ef	5.60g	259.25
T_{12}	BA+KIN	1.50+3.00	3.63bc	4.46cd	5.86f	268.51
T_{13}	BA+KIN	2.00+3.00	3.76b	4.50c	6.83d	316.20
T_{14}	BA+KIN	0.50+4.00	3.93a	4.86a	8.43a	390.27
T_{15}	BA+KIN	1.00+4.00	3.63c	4.67b	7.36b	340.74
T_{16}	BA+KIN	1.50+4.00	3.50cde	4.50c	7.13c	330.09
T_{17}	BA+KIN	2.00+4.00	3.36e	4.36de	6.43e	297.68
CV%		-	2.43	1.77	2.31	-
LSD value		-	0.1285	0.1173	0.2032	-

*WAI=Weeks After Inoculation. In a column, mean values with the same letters are not statistically different from each other at 5% probability by DMRT.

4.3. Sub-experiment 3. Effect of IAA and IBA on root induction potentiality of micro propagated shoots in Betel vine.

To develop root in the regenerated shoots, they were excised and transferred to rooting media supplemented with IAA and IBA separately. The results of experiment are discussed separately under different headings bellow.

4.3.1. Effect of IAA on root formation

The result of the effect of different concentrations of IAA has been presented under following headings with Figure (5-7), Table 6. Padhan (2015) reported micro shoots were inoculated to MS basal media supplemented with 0.5 mg/L IAA was suitable for profuse rooting.

4.3.1.1. Days to root induction

Significant variation was observed among different concentrations of IAA on days to root induction. The maximum days to root induction was recorded in control treatment (35 days) and minimum days (16) was required in 2.0 mg/l of IAA concentration (Figure 5).

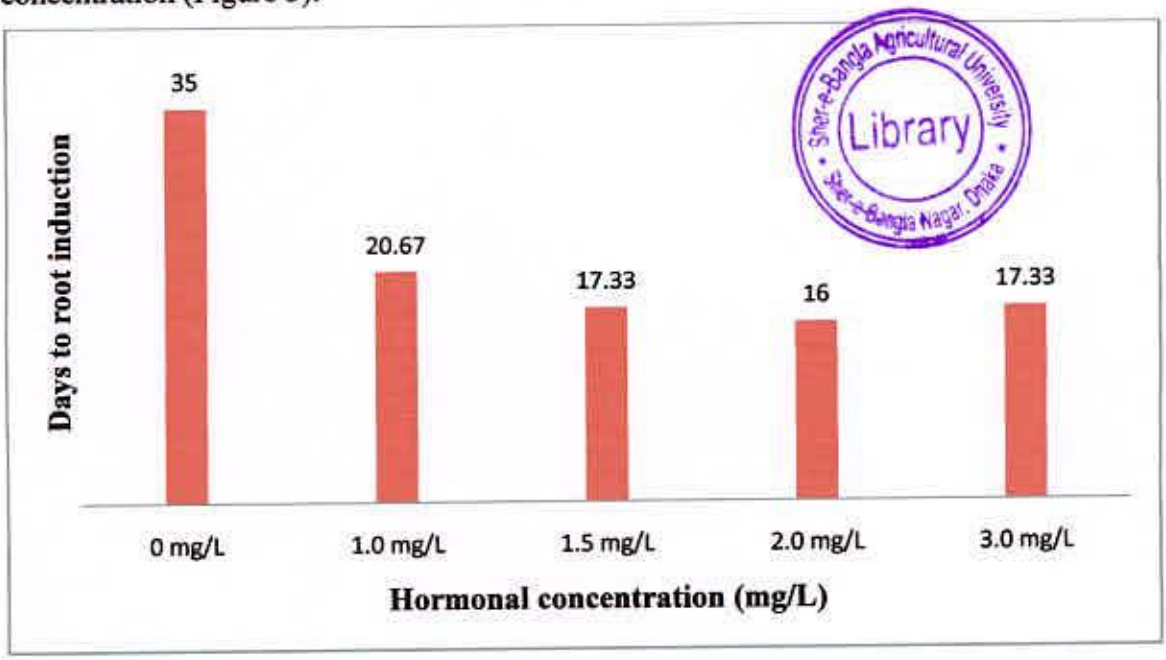

Figure 5. Effect of IAA on days to root induction in Betel vine.

4.3.1.2. Percent of explants showing root induction

Variations were observed among different concentrations of IAA on percent of explants showing root induction. The highest percentage (80%) of root induction was recorded with 2.0 mg/L IAA, whereas the lowest percentage (20%) of root induction was recorded in control condition (Figure 6). Domínguez *et al.* (2006) noticed 100% of elongated shoots were successfully rooted on half strength MS supplemented with 2.0 mg/L IAA.

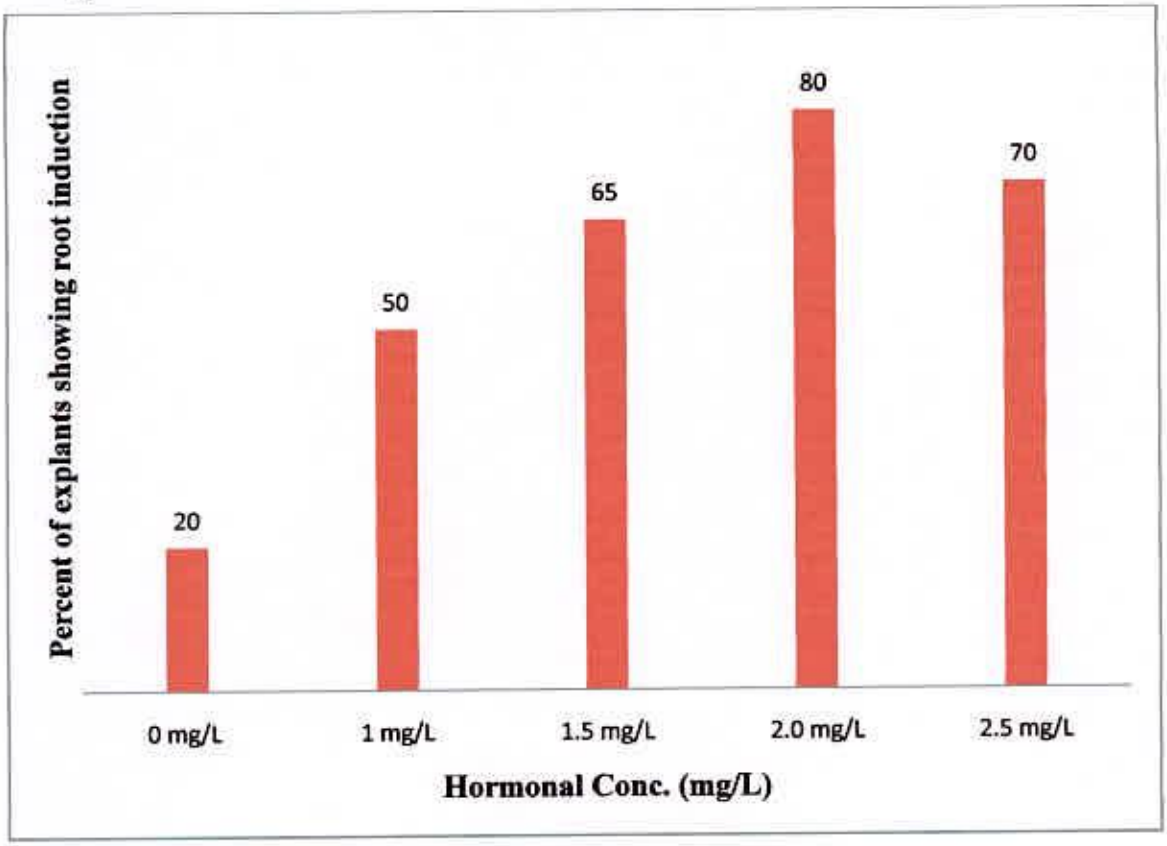

Figure 6. Effect of IAA on percent of explants showing root induction in Betel vine.

4.3.1.3. Number of root per explant

There was significant influence of different concentrations of IAA on the number of root per explant. Data were recorded after 3, 5 and 8 weeks of culture on MS media. The results have been presented in Table 6. The treatment 2.0 mg/L IAA gave the highest number of root 4.70, 7.83 and 13.07 at 3WAI, 5WAI and 8WAI respectively whereas the lowest number of root 1.13 and 2.73, 4.86 at 3WAI, 5WAI and 8WAI respectively was found with hormone free media (Table 6). Considering the percent increase of number of root per explant over control, 2.0 mg/L IAA showed the highest increase (268.93%) at 8WAI. The minimum increase over control (194.95%) was observed in 1.0 mg/L IAA at 8WAI.

Table 6. Effect of IAA on number of root in Betel vine.

Treatments	Name of the phytohormone	Phytohormone concentration (mg/L)	Number of root per explant			
			No. of root per explant (3 WAI)	No. of root per explant (5 WAI)	No. of root per explant (8 WAI)	% increase over control (8 WAI)
T_1	Control	0.0	1.13e	2.73e	4.86e	-
T_2	IAA	1.0	1.73d	4.93d	9.47c	194.85
T_3	IAA	1.5	3.06b	6.43b	11.67b	240.12
T_4	IAA	2.0	4.70a	7.83a	13.07a	268.93
T_5	IAA	3.0	3.20c	4.80c	8.13d	167.28
CV %			6.70	7.74	2.27	-
$LSD_{0.05}$			0.3355	0.9223	0.5366	-

*WAI=Weeks After Inoculation. Values in the column are the means of five replicates. In a column, mean values with the same letters are not statistically different from each other at 5% probability by DMRT.

4.3.1.4. Average length of root per explant(cm)

Average length of root per explant (cm) was greatly regulated by the different concentrations of IAA. The maximum average root length (4.23 cm) was obtained from 2 mg/L IAA (Figure 7) and minimum 1.53cm average length of root per explant (cm) was in control.

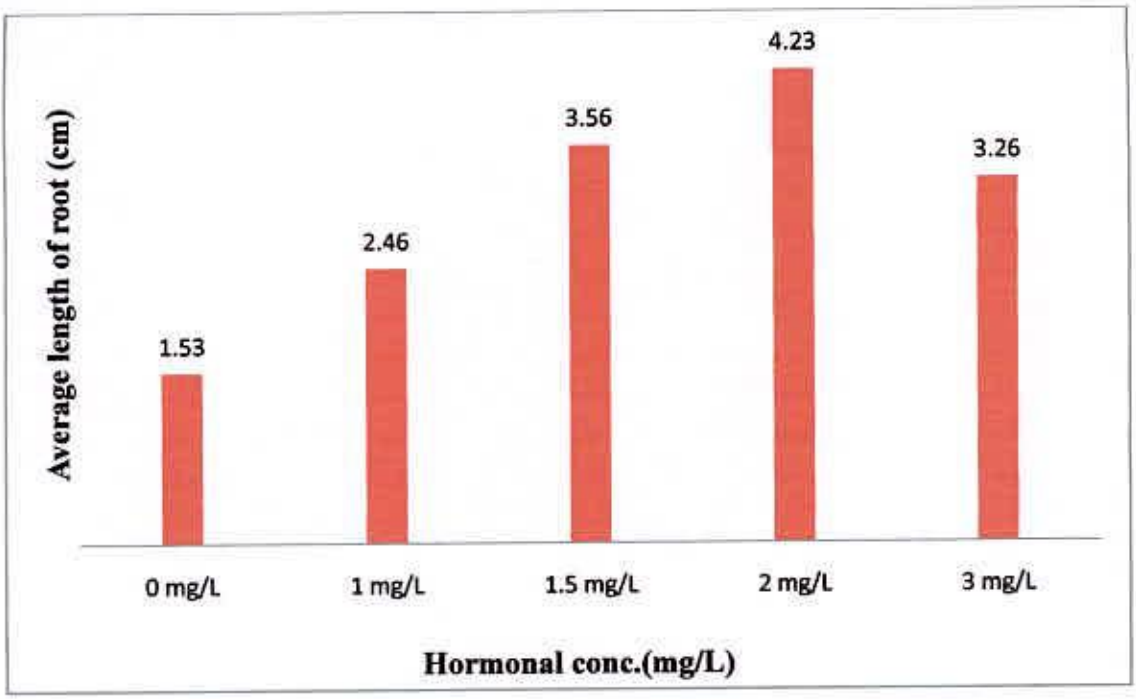

Figure 7. Effect of IAA on average length of root (cm) in Betel vine.

4.3.2. Effect of IBA on root formation

The results of the effect of different concentrations of IBA have been presented under following headings with Figure (8-9), Table (7-8) and Plate 7-9. Hussain *et al.* (2011) mentioned the plantlets formed were rooted best on 1.5 mg/L IBA.

4.3.2.1. Days to root induction

Significant variations were observed among different concentration of IBA on days to root induction. The maximum days to root induction was recorded in control (35 days) and minimum days (14.33) required at 3.0 mg/l of IBA (Figure 8).

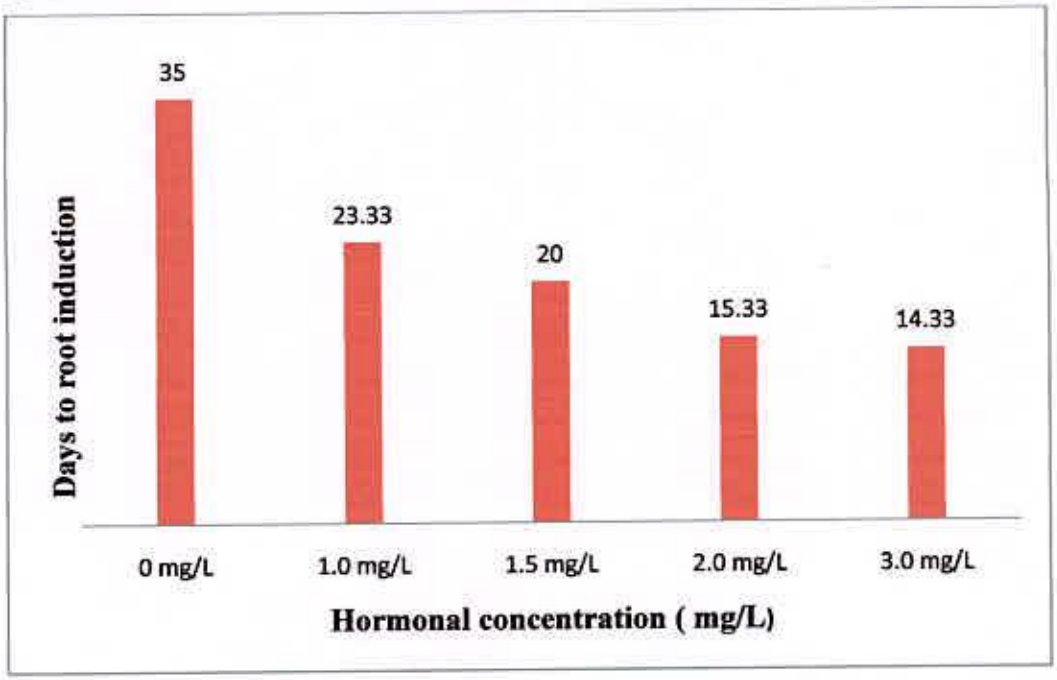

Figure 8. Effect of IBA on days to root induction in Betel vine.

Plate 7. Root initiation start at 14.33 DAI in Betel vine when MS supplemented with 3.0 mg/L IBA.

4.3.2.2. Percent of explants showing root induction

Significant variations were observed among different concentrations of IBA on percent of explants showing root induction. The highest percentage (90%) of root induction was recorded with 2.0 mg/L IBA, whereas the lowest percentage (20%) of root induction was recorded in media lack of plant growth regulator (Figure 9). Datta *et al.* (2002) observed 98% root induction on MS media supplemented with 2.46 M indole butyric acid (IBA).

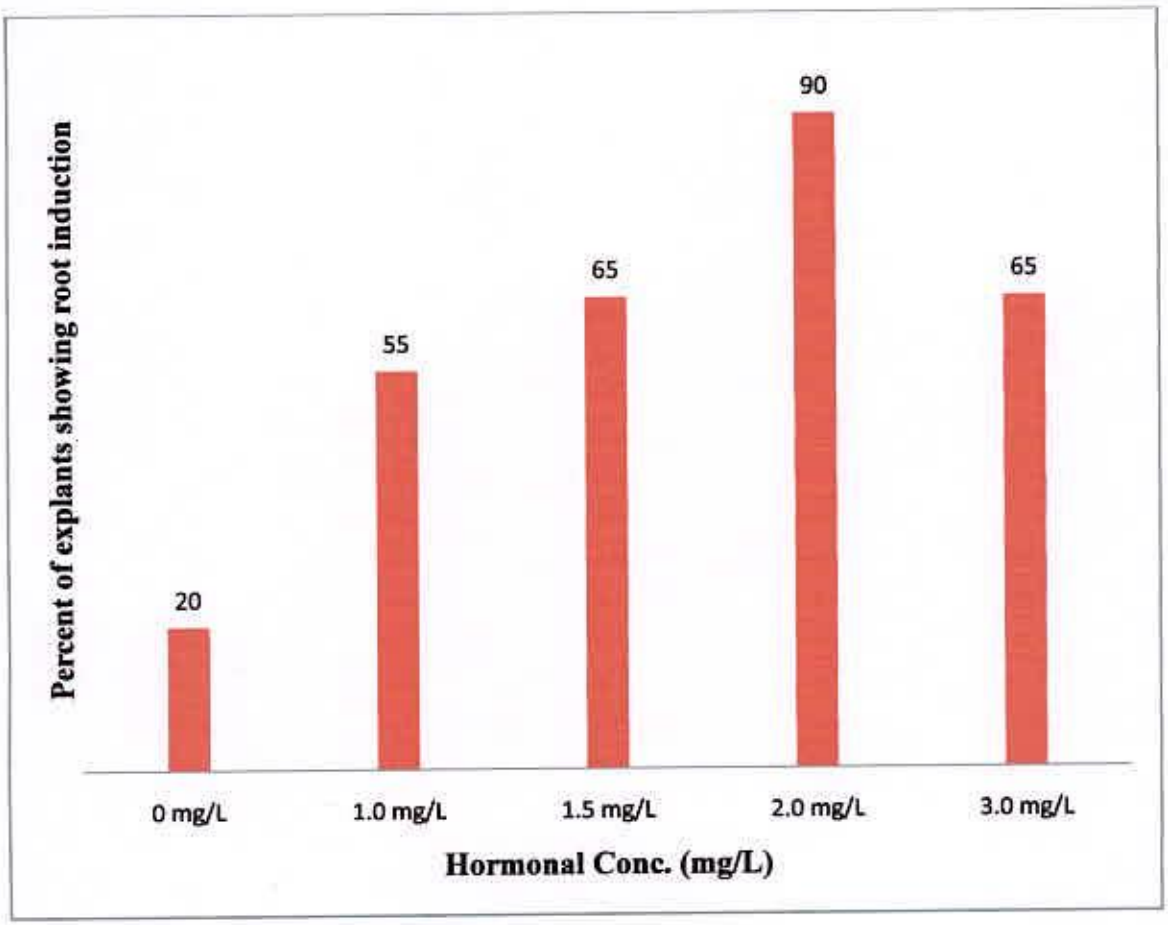

Figure 9. Effect of IBA on percent of explants showing root induction in Betel vine.

4.3.2.3. Number of root per explant

There was significant influence of different concentrations of IBA on the number of roots per explant. Data were recorded after 3, 5 and 8 weeks of culture on MS media. The results have been presented in Table 7. The treatment 3.0 mg/L IBA gave the highest number of roots (6.53, 14.87 and 22.40 at 3WAI, 5WAI and 8WAI) respectively whereas the lowest number of roots (1.13 and 2.73, 5.86 at 3WAI, 5WAI and 8WAI) respectively was found with hormone free media (Table 7). Considering the percent increase of number of root per explant over control, 3.0 mg/L IBA showed the highest increase (296.29%) at 8WAI. The minimum increase over control (201.64%) was observed in 1.0 mg/L IBA at 8WAI. Anand *et al.* (2000) observed the shoot rooted on one-quarter MS supplemented with 2.5 mg/L IBA in the dark produced eight roots per explant.

Table 7. Effect of IBA on number of roots in Betel vine.

Treatments	Name of the phytohormone	Phytohormone concentration (mg/L)	Number of roots per explant			
			No. of root per explant (3 WAI)	No. of root per explant (5 WAI)	No. of root per explant (8 WAI)	% increase over control (8 WAI)
T_1	Control	0.0	1.13 e	2.73e	4.86e	-
T_2	IBA	1.0	3.73d	5.86d	9.80d	201.64
T_3	IBA	1.5	4.86c	7.66c	11.67c	240.12
T_4	IBA	2.0	5.07b	9.87b	13.37b	275.10
T_5	IBA	3.0	6.53a	10.87a	14.40a	296.29
CV %			3.87	2.16	3.56	-
$LSD_{0.05}$			0.2989	0.3593	0.9864	-

*WAI=Weeks After Inoculation. In a column, mean values with the same letters are not statistically different from each other at 5% probability by DMRT.

Plate 8. Effect of IBA on number of root per explant in Betel vine when MS supplemented with 3.0 mg/L IBA at 8WAI.

4.3.2.4. Average length of root per explant (cm)

Average length of root per explant (cm) was greatly regulated by the different concentrations of IBA. The maximum average root length (7.16 cm) was obtained from 3 mg/L IBA (Table 8) and minimum 1.53 cm average length of root per explant (cm) was in control. Considering the percent increase of average length of root over control, 3.0 mg/L IBA showed the highest increase (467.97%) at 8WAI. The minimum increase over control (204.57%) was observed in 1.0 mg/L IBA at 8WAI. Anand *et al.* (2000) observed that the shoot rooted on one-quarter MS supplemented with 2.5 mg/L IBA in the dark produced roots with an average root length of 3.36 cm.

Table 8. Effect of IBA on average length of root per explant (cm) in Betel vine.

Treatments	Name of the Phytohormnes	Phytohormones Concentration (mg/L)	Average length of root per explant (cm)	% increase over control
T_1	Control	0.0	1.53e	-
T_2	IBA	1.0	3.13d	204.57
T_3	IBA	1.5	4.86c	317.64
T_4	IBA	2.0	5.76b	376.47
T_5	IBA	3.0	7.16a	467.97
CV%		-	8.07	-
LSD value		-	0.6585	-

In a column, mean values with the same letters are not statistically different from each other at 5% probability by DMRT.

Plate 9. Effect of IBA on length of root per explant in Betel vine when MS supplemented with 3.0 mg/L IBA at 8WAI.

4.3.3. The comparative performance of IAA and IBA on root formation

The comparative performance of two growth hormones revealed variation among different parameters under study. The result has been presented in Figure 10-13.

4.3.3.1. Days to root induction

Hormonal concentration has significant level of variation on days for root induction. The maximum 35 days to root induction was required in media lack of growth regulator. Minimum 16 days was noticed 2.0 mg/L IAA and 14.33 days was required by 3.0 mg/L IBA (Figure 10).

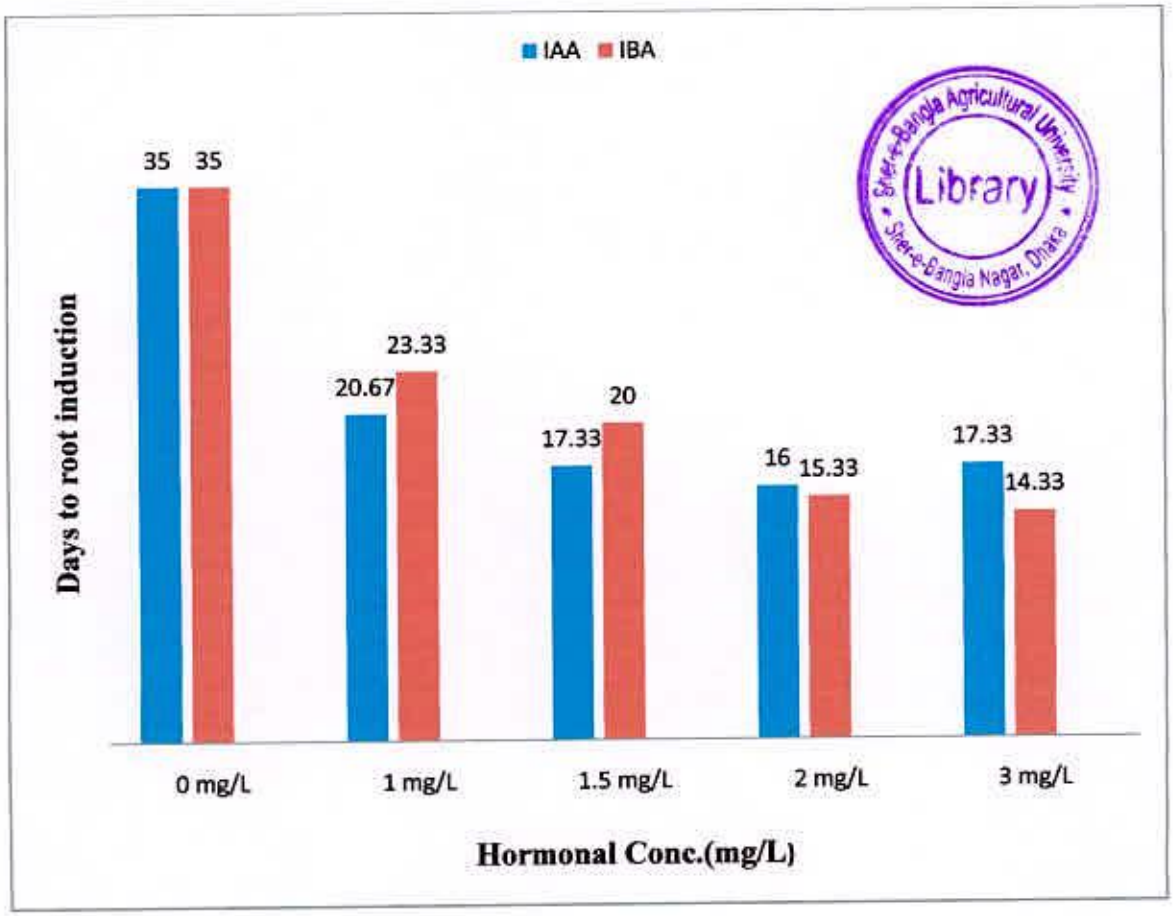

Figure 10. Effect of IAA and IBA on days to root induction

4.3.3.2. Percent of explants showing root induction

There were comparable variations among growth regulators on percent of explants showing root induction. The results have been shown in Figure 11. The highest percentage (90%) of root induction was recorded with 3.0 mg/L IBA, whereas 80% root induction was found in 2.0 mg/L IAA. The lowest percentage (20%) of root induction was recorded in control treatment (Figure 11).

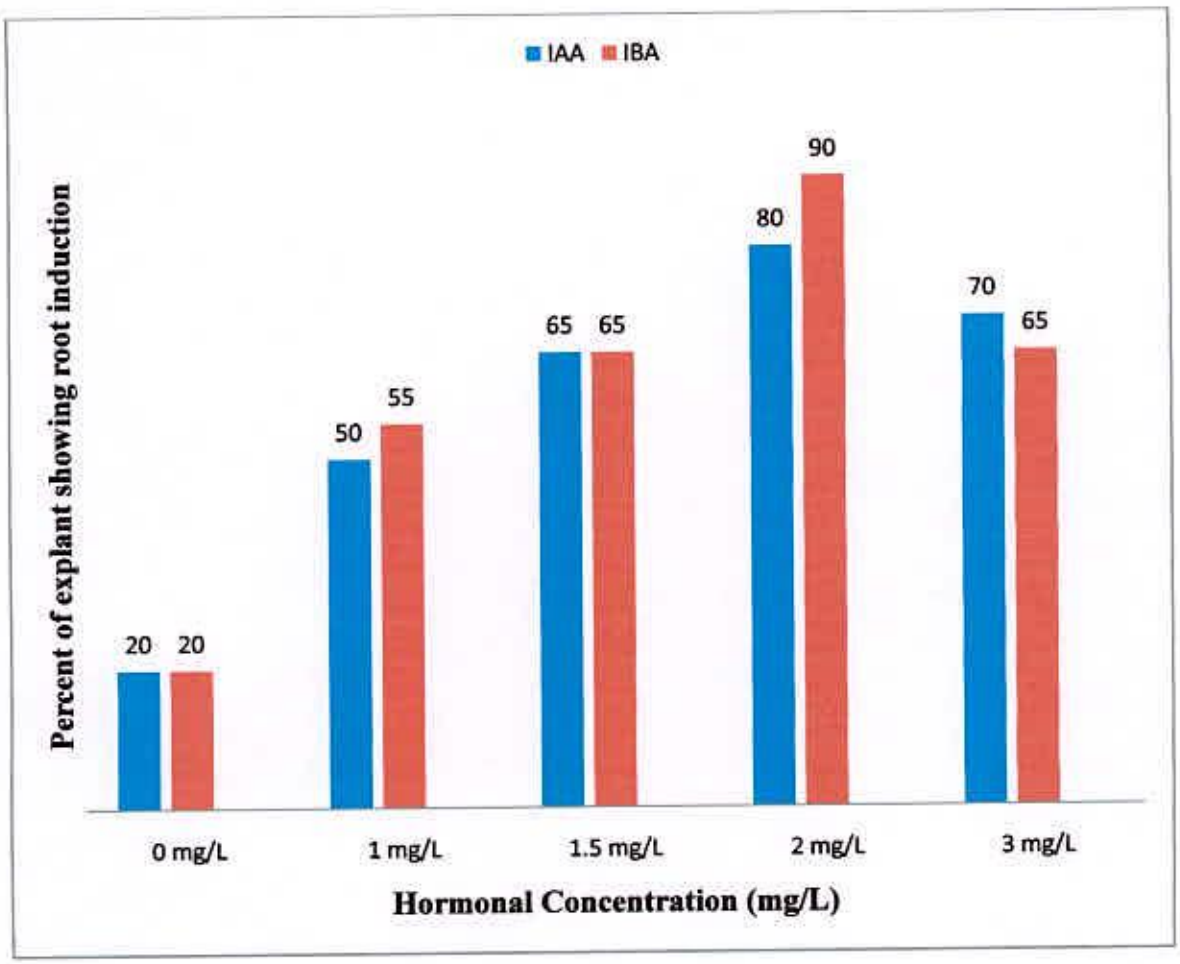

Figure 11. Effect of IAA and IBA on percent of explants showing root induction.

4.3.3.3. Number of root per explant 8 WAI

To check the response and effectiveness of IAA and IBA on the number of root a range of treatment (0, 1.0, 1.5, 2.0 and 3.0) were applied and less significant variation were observed during data recording at 8WAI. Only 3.0 mg/L of IAA and IBA gave significant variation (figure 12). The highest number of root (14.40) per explant was recorded in 3.0 mg/L IBA at 8WAI, and 13.07 in case of 2.0 mg/L IAA. The minimum number of root (4.86 at 8 WAI) was obtained in control (Figure 12).

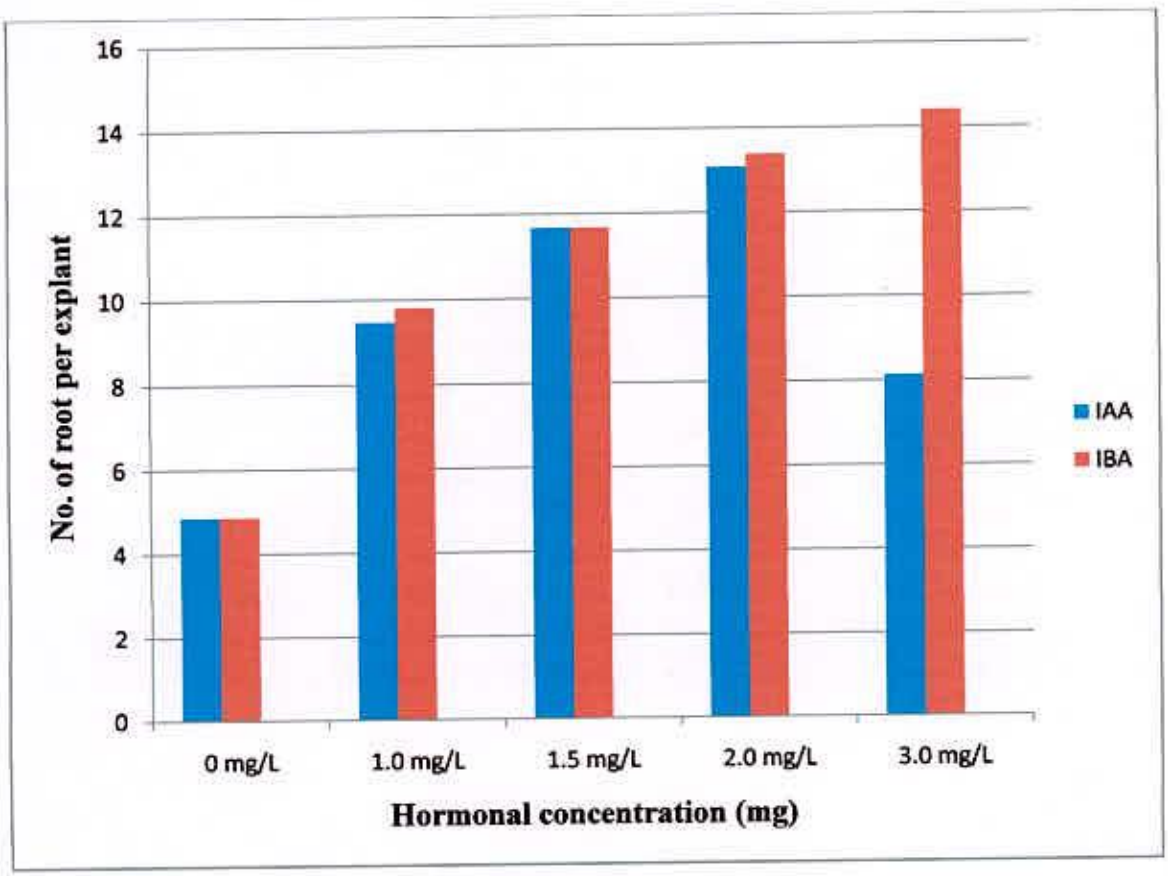

Figure 12. Effect of IAA and IBA on number of root per explant.

4.3.3.4. Average length of root per explant (cm) at 8 WAI

Average length of root per explant (cm) was greatly regulated by the different concentrations of both IAA and IBA. The maximum average root length (7.16 cm) was obtained from 3.0 mg/L IBA (Figure 13) and 4.23 cm in case of 2.0 mg/L IAA (Figure 14). The minimum 1.53 cm average length of root per explant (cm) was in control.

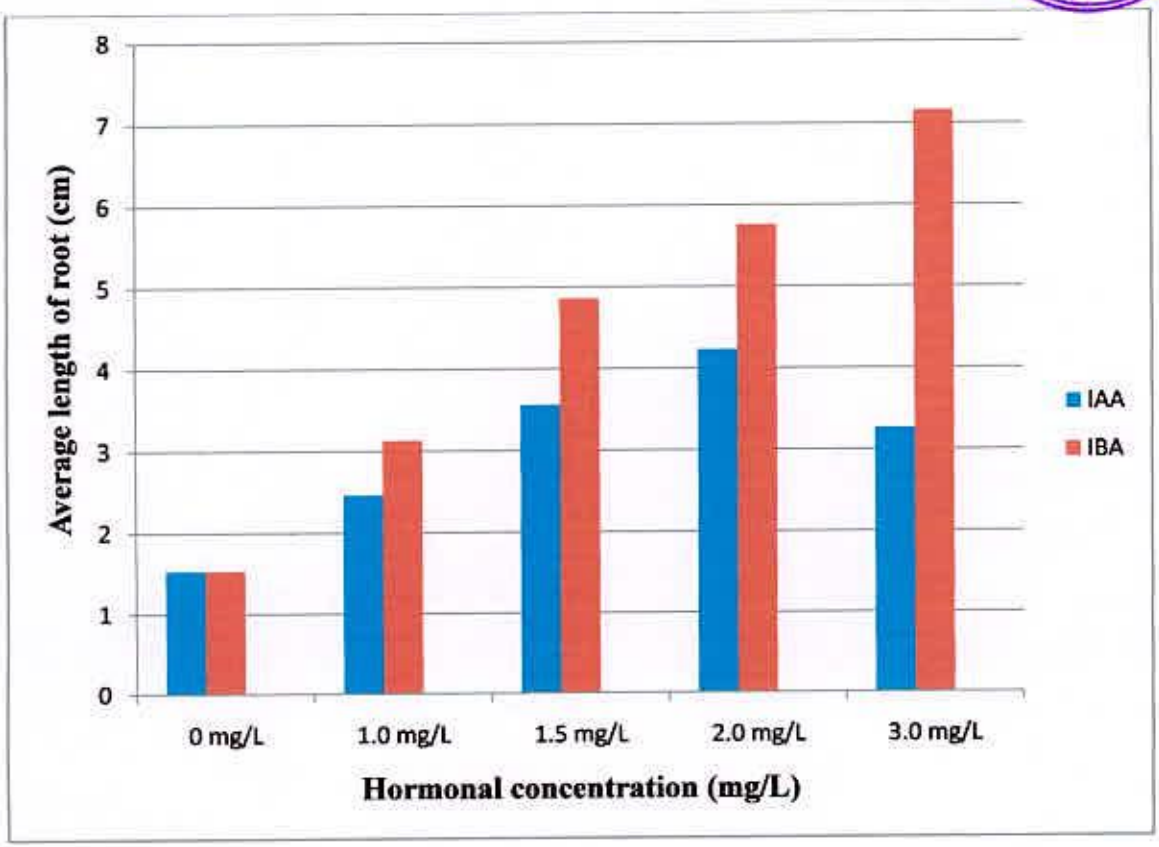

Figure 13. Effect of IAA and IBA on average length of root per explant.

4.4. Experiment 3. *Ex vitro* acclimatization and establishment of plantlets on soil

The results of acclimatization or "hardening-off' have been presented in Table 9 and Plate 10. After 8 weeks of culture on rooting media, the plantlets were taken for acclimatization.

Table 9. Survival rate of *in vitro* regenerated plantlet of Betel vine.

Acclimatization	No. of plantlet transplanted	No. of plantlet survived	Survival rate (%)
In growth chamber	20	18	90
In shade house	18	15	83
In natural condition	15	10	64

The results of acclimatization showed that the 90% of plantlets survived to growth chamber (Table 9). Then the plantlets were shifted to shade house with less humidity (70% RH) and indirect sunlight. In the shade house, the top of the pots were covered with transparent plastic sheet and grew at room temperature with periodic irrigation (2 days interval). In these conditions, the 83% of the plantlets showed survival (Table 9). Then the plantlets were transferred to the soil following depoting and potting into different pots of bigger size. In open atmosphere, survival rate was 64% (Table 9). Anand *et al.* (2000) reported that in natural condition 75% plantlets survived. Padhan (2015) observed 90% plantlets survival in soil in shade house.

A B

Plate 10. Acclimatization of regenerated plantlets (A) in growth chamber and (B) in natural condition.

Finally the overall outline of this experiment was that it was conducted to evaluate the *in vitro* propagation of betel vine using nodal segment on MS media supplemented with different concentrations of BA, BA+KIN, IAA and IBA. Similar statement of role of tissue culture, among plant growth regulator, cytokinin in culture media is the most important factor for shoot proliferation (Liao *et al.*, 2004, Mamidala and Nanna, 2009, Hoque, 2010). Applying of plant growth regulator is also important for Rooting in culture (Feng *et al.*, 2000, Hongzhi, 2000). Some researchers reported presence of both of auxin and cytokinin important for shoot proliferation (Rout *et al.*, 2001, Velcheva *et al.*, 2005).

Betel vine (*Piperacae.*) was propagated by direct or indirect organogenesis using various explants including, shoot tip (Zuraida *et al.*, 2015, Datta *et al.*, 2002), leaf (Dominguez *et al.*, 2006, Balbuena, 2009), nodal segment (Parida *et al.*, 2011, Bhat *et al.*, 1992, Padhan, 2015). Present study finding showed that nodal segment was suitable to induce direct multiple shoot regeneration. Initially shoot tip was used as explant, result of which has not been mentioned. For direct shoot regeneration, nodal segment showed good response.

Auxin and cytokinin are the most common plant growth regulators used *in vitro* regeneration (George and Debergh, 2008). The cytokinin type and concentrations are important factors for successful *in vitro* multiplication. The best shoot proliferation response of nodal explant was observed with a cytokinin combination of benzyladenine and kinetin in *Piperacae* (Anand *et al.*, 2000). Auxin play important role in root induction in tissue culture plantlets. In present study MS media supplemented with sole dose of BA (0.5, 1.0, 1.5 and 2.0 mg/L), IAA (1.0, 1.5, 2.0 and 3.0 mg/L) and IBA (1.0, 1.5, 2.0 and 3.0 mg/L) were used. The study results revealed that BA with 2.0 mg/L gave the best response taking minimum days to shoot induction, highest multiple shoots and leaves, which similar with results of 1.5 mg/L BA. Similarly IAA with 2.0 mg/L was the best and took least time for root induction, produce maximum roots with highest root length. But IBA with 3.0 mg/L was the best and took least time for root induction, produce maximum roots with highest length. That is the best response was achieved in higher moderate doses of both cytokinin and auxin for both shoot and root formation. This might be due to the fact that lower hormone doses are unable to fully support the growth and development. Sometime excess higher dose render unknown adverse or toxic effect hampering desired growth.

Narayanaswamy (1977) reported that an excess of growth regulators in the culture media, might lead to genetic, physiological and morphological change resulting in a reduction of the proliferation rate *in vitro*.

The cytokinin combination of BA and KIN are most effective than sole dose of cytokinin in *Piperacae.* (Anand *et al.*, 2000, Datta *et al.*, 2002, Padhan, 2015). The study result revealed that, MS media supplemented with 1.0 mg/L BA+ 3.0 mg/L KIN showed significant superiority compared to other because it took least number of days to shoot induction, maximum shoots and leaves. Present research results are consistent with the previous reports regarding the positive effect of BA and KIN on shoot proliferation. This difference in hormone concentration highlights the importance of exogenous supply of growth regulators to achieve higher multiplication rate.

Successful transfer of *in vitro* regenerated plantlets to soil with good survival rates and free of any abnormalities are prerequisites for micropropagation which necessitate proper acclimatization. A heterotrophic mode of nutrition and poor mechanism for water loss control further renders. Micropropagated plants vulnerable to transplantation shocks. In this way acclimatization was mode in betel vine was proved appropriate as highest 64% survival rate was achieved. None of the regenerated plantlets showed morphological abnormalities.

Chapter V
Summary

CHAPTER V

SUMMARY

The experiment entitled "*In vitro* regeneration of Betel vine." was conducted at the Biotechnology Laboratory of Department of Biotechnology, Sher-e-Bangla Agricultural University, Sher-e-Bangla Nagar, Dhaka during the period from July 2014 to April 2015 to investigate the effect of different plant growth regulator on shoot proliferation and root formation along with acclimatization under field condition. Nodal segment of Betel vine. was used as an explant for *in vitro* propagation of Betel vine. The major findings have been presented below.

The combine hormonal treatment 1.0 mg/L BA+ 3.0 mg/L KIN showed minimum days to shoot induction in betel vine. Percent of shoot regeneration also highest in the combine treatment 1.5 mg/L BA+ 3.0 mg/L KIN.

The combination of BA and KIN, the highest number of shoot 2.03, 3.46 and 4.63 at 3WAI, 5WAI and 8WAI respectively were recorded in 1.0 mg/L BA+ 3.0 mg/L KIN.

The maximum average length of shoot (2.36, 2.93 and 3.85 cm at 3WAI, 5WAI and 8WAI) respectively were recorded in 1.5 mg/L BA. Eight weeks after induction maximum number of leaves (4.8) was recorded when media supplemented with 1.0 mg/L BA+ 3.0 mg/L KIN, whereas minimum number of leaves (2.1) was counted in control.

The maximum 35.33 days was required for root induction in media without plant growth regulator, minimum 14.33 days recorded in 3.0 mg/L IBA.

The highest number of root 4.70, 7.83 and 13.07 at 3WAI, 5WAI and 8WAI respectively were counted in 2.0 mg/L IAA and in case of IBA application the highest number of root 6.53, 10.87 and 14.40 at 3WAI, 5WAI and 8WAI respectively in 3.0 mg/L IBA.

Plantlets were transplanted from culture media to soil in plastic pots under controlled environment, where the survival rate was 90%. The hardened plantlets were transferred to open field where the survival rate was 64%.

Chapter VI
Conclusion

CHAPTER VI

CONCLUSION

Following conclusions can be made from the present study:

i. A micropropagation protocol has been developed in Betel vine

ii. The treatment 2.0 mg/L BA response excellent in shoot induction and 2.5 mg/L IBA showed better performance in root development in Betel vine.

iii. IBA is better than IAA for rooting in Betel vine at *in vitro* condition.

iv. Combined effect of BA+KIN seems to be better than individual effect of BA for shoot formation. The treatment 1.0 mg/L BA+ 3.0 mg/L KIN revealed the best treatment for shoot induction.

Recomendations

RECOMMENDATIONS

Following recommendations could be addressed based on the present experiment:

i. Further research can be carried out on more specific hormonal combination of BA, KIN, IAA, IBA or any other phytohormone.
ii. Callus induction could be practiced for more shoot proliferation.
iii. Future experiment should be carried on different type of genotype of Betel vine.
iv. Fixed survival rate of in vitro plant should be studied and compare to normal planting.
v. Yield potentiality between micro propagated and vegetative propagated plants can be studied.

Chapter VII
References

CHAPTER VII
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Appendices

APPENDICES

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

Components	Concentrations (mg/L)	Concentrations
Micro Elements	**mg/L**	**µM**
$CoCl_2.6H_2O$	0.025	0.11
$CuSO_4.5H_2O$	0.025	0.10
Fe Na EDTA	36.70	100.00
H_3BO_3	6.20	100.27
KI	0.83	5.00
$MnSO_4.H_2O$	16.90	100.00
$Na_2MoO_4.2H_2O$	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**	**µM**
Glycine	2.00	26.64
Myo-Inositol	100.00	554.94
Nicotinic acid	0.50	4.06
Pyridoxine HCl	0.50	2.43
Thiamine HCl	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 variance on days to shoot induction with BA

	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability	CV%	$LSD_{0.05}$
Between	4	367.333	91.833	196.786	0.0000	2.36	1.243
Within	10	4.667	0.467				
Total	14	372.000					

Appendix III . Analysis of variance on number of shoot per explant at 3WAI with BA

	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability	CV%	$LSD_{0.05}$
Between	4	0.071	0.018	13.250	0.0005	3.26	0.05753
Within	10	0.013	0.001				
Total	14	0.084					

Appendix IV . Analysis of variance on number of shoot per explant at 5WAI with BA

	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability	CV%	$LSD_{0.05}$
Between	4	0.231	0.058	12.357	0.0007	5.20	0.1286
Within	10	0.047	0.005				
Total	14	0.277					

Appendix V . Analysis of variance on number of shoot per explant at 8WAI with BA

	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability	CV%	$LSD_{0.05}$
Between	4	1.051	0.263	56.286	0.0000	3.91	0.1286
Within	10	0.047	0.005				
Total	14	1.097					

Appendix VI . Analysis of variance on length of shoot at 3WAI with BA

	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability	CV%	$LSD_{0.05}$
Between	4	0.387	0.097	20.714	0.0001	3.87	0.1286
Within	10	0.047	0.005				
Total	14	0.433					

Appendix VII . Analysis of variance on length of shoot at 5WAI with BA

	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability	CV%	$LSD_{0.05}$
Between	4	0.689	0.172	25.850	0.0000	3.32	0.1522
Within	10	0.067	0.007				
Total	14	0.756					

Appendix VIII . Analysis of variance on length of shoot at 8WAI with BA

	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability	CV%	$LSD_{0.05}$
Between	4	1.436	0.359	59.833	0.0000	2.81	0.1409
Within	10	0.060	0.006				
Total	14	1.496					

Appendix IX . Analysis of variance on number of leaves at 8WAI with BA

	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability	CV%	$LSD_{0.05}$
Between	4	7.143	1.786	167.406	0.0000	1.89	0.1908
Within	10	0.107	0.011				
Total	14	7.249					

Appendix X . Analysis of variance on days to shoot induction with combination of BA and KIN

	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability	CV%	$LSD_{0.05}$
Between	16	680.706	42.544	48.217	0.0000	3.83	1.558
Within	34	30.000	0.882				
Total	50	710.706					

Appendix XI . Analysis of variance on number of shoot per explant at 3WAI with combination of BA and KIN

	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability	CV%	$LSD_{0.05}$
Between	16	4.693	0.293	59.830	0.0000	4.68	0.1173
Within	34	0.167	0.005				
Total	50	4.859					

Appendix XII . Analysis of variance on number of shoot per explant at 5WAI with combination of BA and KIN

	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability	CV%	$LSD_{0.05}$
Between	16	10.774	0.673	68.685	0.0000	3.88	0.1659
Within	34	0.333	0.010				
Total	50	11.107					

Appendix XIII . Analysis of variance on number of shoot per explant at 8WAI with combination of BA and KIN

	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability	CV%	$LSD_{0.05}$
Between	16	26.210	1.638	278.479	0.0000	2.26	0.1285
Within	34	0.200	0.006				
Total	50	26.410					

Appendix XIV . Analysis of variance on length of shoot at 3WAI with combination of BA and KIN

	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability	CV%	$LSD_{0.05}$
Between	16	17.086	1.068	175.686	0.0000	2.43	0.1285
Within	34	0.207	0.006				
Total	50	17.293					

Appendix XV . Analysis of variance on length of shoot at 5WAI with combination of BA and KIN

	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability	CV%	$LSD_{0.05}$
Between	16	21.253	1.328	260.548	0.0000	1.77	0.1173
Within	34	0.173	0.005				
Total	50	21.426					

Appendix XVI . Analysis of variance on length of shoot at 8WAI with combination of BA and KIN

	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability	CV%	$LSD_{0.05}$
Between	16	124.232	7.764	535.120	0.0000	2.31	0.2032
Within	34	0.493	0.015				
Total	50	124.725					

Appendix XVII . Analysis of variance on number of leaves at 8WAI with combination of BA and KIN

	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability	CV%	$LSD_{0.05}$
Between	16	29.013	1.813	144.496	0.0000	1.66	0.1892
Within	34	0.427	0.013				
Total	50	29.439					

Appendix XVIII . Analysis of variance on days to root formation with IAA

	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability	CV%	$LSD_{0.05}$
Between	4	770.667	192.667	115.600	0.0000	6.05	2.349
Within	10	16.667	1.667				
Total	14	787.333					

Appendix XIX . Analysis of variance on number of root per explant at 3WAI with IAA

	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability	CV%	$LSD_{0.05}$
Between	4	23.233	5.808	169.137	0.0000	6.70	0.3355
Within	10	0.343	0.034				
Total	14	23.576					

Appendix XX . Analysis of variance on number of root per explant at 5WAI with IAA

	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability	CV%	$LSD_{0.05}$
Between	4	79.711	19.928	77.640	0.0000	7.74	0.9223
Within	10	2.567	0.257				
Total	14	82.277					

Appendix XXI . Analysis of variance on number of root per explant at 8WAI with IAA

	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability	CV%	$LSD_{0.05}$
Between	4	287.483	71.871	822.946	0.0000	2.27	0.5366
Within	10	0.873	0.087				
Total	14	288.356					

Appendix XXII . Analysis of variance on average length of root at 3WAI with IAA

	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability	CV%	$LSD_{0.05}$
Between	4	13.044	3.261	111.170	0.0000	5.68	0.3098
Within	10	0.293	0.029				
Total	14	13.337					

Appendix XXIII . Analysis of variance on days to root formation at 3WAI with IBA

	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability	CV%	$LSD_{0.05}$
Between	4	858.667	214.667	128.800	0.0000	5.96	2.349
Within	10	16.667	1.667				
Total	14	875.333					

Appendix XXIV . Analysis of variance on number of root per explant at 3WAI with IBA

	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability	CV%	$LSD_{0.05}$
Between	4	48.720	12.180	445.610	0.0000	3.87	0.2989
Within	10	0.273	0.027				
Total	14	48.993					

Appendix XXV . Analysis of variance on number of root per explant at 5WAI with IBA

	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability	CV%	$LSD_{0.05}$
Between	4	249.307	62.327	1584.577	0.0000	2.16	0.3593
Within	10	0.393	0.039				
Total	14	249.700					

Appendix XXVI . Analysis of variance on number of root per explant at 8WAI with IBA

	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability	CV%	$LSD_{0.05}$
Between	4	486.864	121.716	414.000	0.0000	3.56	0.9864
Within	10	2.940	0.294				
Total	14	489.804					

Appendix XXVII . Analysis of variance on average length of root at 8WAI with IBA

	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability	CV%	$LSD_{0.05}$
Between	4	58.556	14.639	111.464	0.0000	8.07	0.6585
Within	10	1.313	0.131				
Total	14	59.869					