IN VITRO PLANT REGENERATION OF *Aloe vera* L.

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CERTIFICATE

This is to certify that thesis entitled, **"*IN VITRO* PLANT REGENERATION OF *Aloe vera* L."** *submitted to the Faculty of* **AGRICULTURE,** *Sher-e-Bangla Agricultural University, Dhaka, in partial fulfillment of the requirements for the degree of* **MASTER OF SCIENCE** *in* **BIOTECHNOLOGY**, *embodies the result of a piece of bona fide research work carried out by* **MD. ALAUDDIN,** *Registration No.* **07-02383** *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.

Date: JUNE, 2014
Place: Dhaka, Bangladesh

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DEDICATED TO
MY
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
Int.	:	International
2-ip	:	2-isopentenyladenine
J.	:	Journal
Mol.	:	Molecular
mg/L	:	Milligram per litre
µM	:	Micromole
MS	:	Murashige and Skoog
PGRs	:	Plant Growth Regulators
Res.	:	Research
Sci.	:	Science
TDZ	:	Thidiazuron
PVP	:	Polyvinylpyrrolidone
PLB	:	Protocorm-like bodies
CV	:	Co-efficient of Variation
^{o}C	:	Degree Celsius
etc.	:	Etcetera
WAI	:	Weeks After Inoculation

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The Author

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BY

MD. ALAUDDIN

ABSTRACT

The present research was carried out on *Aloe vera* L. in Biotechnology Laboratory of the Department of Biotechnology, Sher-e-Bangla Agricultural University, Sher-e-Bangla Nagar, Dhaka-1207 during the period from September, 2013 to July, 2014 to evaluate the effect of different plant growth regulators on multiple shoot proliferation and root formation along with acclimatization for *in vivo* survival and finally to develop a viable micropropagation protocol. Shoot tip of young lateral shoots (sucker) were used as the explants, which were sterilized using freshly prepared 0.1% $HgCl_2$ solution with few drops of Tween-20. After sterilization, explants were inoculated in MS media supplemented with either singly or in combination of the Benzylamino purine (BAP), Kinetine (KIN), Indolebutyric acid (IBA), Naphthaleneacetic acid (NAA). The highest percentage of shoot proliferation (85%) and minimum days to shoot induction (13.6 days) were achieved on MS medium containing 1.0 mg/L BAP + 0.5 mg/L IBA. The highest 14.8 shoots and 24.4 leaves per explant were also observed in the same media composition. But the highest average length of shoots (9.62 cm) was recorded from media containing 1.5 mg/L BAP + 1.0 mg/L NAA. The highest percentage (95%) of rooting was obtained in media treated with 3.0 mg/L IBA along with maximum 12.4 roots per explant. But the highest (10.24 cm) average length of root was observed in 2.0 mg/L IBA. The highest (5.374 mm) diameter of root and minimum days to root induction (10.6 days) were noticed on the same culture media supplemented with 2.5 mg/L NAA. In regenerated plantlets, 100% survival in growth chamber conditions and 88% survival under shade house and 90% survival in open atmosphere were achieved. Micropropagation was found to be very effective and promising method in the propagation of *Aloe vera* and this protocol can be a useful tool for proliferation of *Aloe vera*.

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CHAPTER I
INTRODUCTION

Aloe vera Linn. is commonly known as “Lily of Desert,” “Medicinal Plant,” “Burn Plant,” “First Aid Plant,” “Miracle Plant,” and “Ghritkumari” in Ayurveda (Tanabe and Horiuchi, 2006). Aloe is the mostly used medicinal plant that belongs to the family Liliaceae but it is *Aloe barbadensis* which is commonly called *Aloe vera*. The name *Aloe vera* derives from the Arabic word “Alloeh” meaning “shining bitter substance,” while “vera” in Latin means “true.”About 2000 years ago, the Greek scientists regarded *Aloe vera* as the universal panacea. The Egyptians called Aloe “the plant of immortality.”*Aloe vera* has been used for medicinal purposes in several cultures for millennia: Greece, Egypt, India, Mexico, Japan and China. Supposedly Alexander the Great in 333 B.C occupied the island of Socotra in the Indian Ocean for its famed Aloe supplies, needed to treat his wounded soldiers (Atherton, 1998).

Aloe vera is native to North Africa, the Mediterranean region of southern Europe, and to the Canary Islands. It is now cultivated throughout the West Indies, tropical America, and the tropics in general (Ross, 2003). There are about 300 species of genus *Aloe* grown around the world. Common species of *Aloe* are *Aloe barbadensis, Aloe saponaria*, *Aloe chinensis, Aloe vera*, *Aloe arborescens, Aloe variegata*, *Aloe forex*, *Aloe latifolia*, of these most popular is *Aloe barbadensis* and *Aloe aborescens.* However, only *Aloe barbadensis* Mill. is grown now a day commercially.

Aloe vera is a monocotyledonous plant (Ali & Qaiser, 2005). It is a perennial xerophytic plant. The plant has triangular, lanceolate, green to grey green colour, thick and fleshy, leaves with serrated edges, yellow tubular flowers and fruits that contain numerous seeds. It spreads by offsets and root sprouts. The flowers are produced in an inflorescence 90 cm tall, each flower is pendulous, with a yellow tubular corolla 2-3 cm and hermaphrodite. The yellow periniath is divided into 6 lobes, about 2.5 cm long, with scattered bracts. Each flower has 6 protruding stamens and three ovary with long style. The plant prefers light (sunny weather), requires well-drained soil and even can grow in nutritionally poor soil.

Aloe vera is a very important indigenous medicinal herb which is used worldwide in cosmetic and pharmaceutical industry. Each leaves is composed of three layers: 1) an inner clear gel that contains 99% water and rest is made of glucomannans, amino

acids, lipids, sterols and vitamins. 2) The middle layer of latex which is the bitter yellow sap and contains anthraquinones and glycosides. 3) The outer thick layer of 15–20 cells called as rind.

More than 160 metabolites are found in *Aloe vera* leaves (Supe, 2007) and among these the most important are barbaloin and homonataloin (Groom & Reynolds, 1987). Besides, out of the 22 amino acids required for the human body, 8 essential amino acids are found in *Aloe vera*. Vitamins namely A, B1, B2, B6, B12, C and E, which the human body cannot prepare by itself, are available in *Aloe vera*. Daily intake of *Aloe vera* gel free from aloin (a yellow sap) is certainly good for health and improves natural immunity.

In Bangladesh, about 80% of the population depend on herbal treatment particularly the Ayurvedic and Unani systems (FAO, 2007). In recent times, *Aloe vera* gel has been used as an active ingredient in hundreds of skin lotions, sun blocks and cosmetics. The gel's use in cosmetics has been increased by claims that it has anti-aging effects similar to vitamin A derivatives. Gel produced in the leaves of this plant makes an excellent treatment for wounds, burns and other skin disorders, placing a protective coat over the affected area, speeding up the rate of healing and reducing the risk of infection (Anshoo *et al*., 2005). The research studies conducted on *Aloe vera* plant have revealed that through strengthening the T-lymphocyte cells of the blood, it is able to heal the wounds and improve immunity (Lee & Kim, 2000). In Bangladesh the street hawker mainly uses it for the preparation of Sherbet (Soft drinks) (Dixie *et al*., 2003).

The current global trade of processed derivatives of *Aloe vera* is estimated at around US$ 1 billions and is steadily increasing. IASC (2004) estimated the industry size as *Aloe vera* raw material $65-80 million and finished products containing *Aloe vera* - $110 billion dollars. A market study on medicinal plants conducted by Dixie *et al.* (2003) revealed that 1000 tonnes of fresh *Aloe vera* is used only for self-consumption per year in Bangladesh. Sales have been steadily growing at around 25 percent year. The market opportunity is estimated at some 90,000 USD (Dixie *et al.*, 2003). Besides, Bangladesh at present spends about TK. 5000 million on the import of herbs and herbal extracts to make medicine. At present the annual average size of the export

market for herbal products worldwide as raw materials is some US$ 62 billion. The market size is projected to expand to around $5 trillion by 2050 (FAO, 2007).

Despite of its enormous potentiality in both domestic and international market, the cultivation of *Aloe vera* in Bangladesh is very limited. It is primarily grown around Natore district and is confined in a small pocket of other areas of the country (Dixie *et al*., 2003). So there is a large prospect for export by cultivating medicinal plants and herbs with special emphasize on *Aloe vera*.

Naturally *Aloe vera* is propagated vegetatively and the multiplication rate is very slow. 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. The symptoms are hardly detectable and the yield and quality decreases. On the other hand, sexual reproduction of *Aloe vera* by means of seeds is limited due to presence of wide spread male sterility (Kalimuthu *et al.*, 2010).

Due to the huge utilization in pharmaceutical, cosmetic and food industries (Eshun and He, 2004; Botes *et al.*, 2008; Grace *et al.*, 2008; Bedini *et al.*, 2009; Rodriguez *et al.*, 2010; Chen *et al.*, 2012; Lad and Murthy, 2013; Zapata *et al.*, 2013), the demand for quality planting material of *Aloe vera* is increasing day-by-day as mentioned above.

Hence, an alternative method is time demanding. *In vitro* technique offers a possibility to solve these problems. Compared 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). Campestrini *et al.* (2006) reproduced 4,300 plantlets from 20 explant, over a 6 month period by using *in vitro* techniques.

Shoot tip, axillary bud, meristem, underground stem and inflorescence have been used as explant (Liao *et al.*, 2004; Velcheva *et al.*, 2005). 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 (Aggarwal and Barna, 2004; Liao *et al.*, 2004; Mamidala and Nanna, 2009; Jafari and Hamidoghli, 2009; Hoque, 2010). Applying of plant growth regulators is necessary for rooting in culture (Abrie and Staden, 2001; Feng *et al.*, 2000; Hongzhi, 2000). Some researchers reported presence of both of auxin and cytokinin necessary for shoot proliferation (Rout *et al.*, 2001; Velcheva *et al.*, 2005). Ali *et al.* (2009) reported the optimum combination of cytokinins and auxin critical to shoot regeneration. Acclimatization of plantlets in the pots containing a mixture of sand and soil under greenhouse conditions with 70-90% moisture is suitable for tissue cultured plantlets survival (Natali *et al*., 1990; Hirimburegama and Gamage, 1995).

So, mass propagation of uniform, healthy plants through tissue culture is the only viable technique for production of large numbers of clonal plants in a short time. Though several attempt were taken for last few decades to develop tissue culture systems of *Aloe spp*. (de Oliveira and Crocomo, 2009; Das *et al.,* 2010; Gantait *et al.,* 2011; Rathore *et al.,* 2011; Amoo *et al.,* 2012, 2013), but still the efficient regeneration protocols are requisite to develop a rapid, less expensive, efficient and easy method of micropropagation of *Aloe vera*.

In this consequence, a new composition of growth regulators for rapid and efficient micropropagation of *Aloe vera* using shoot tip of young offsets as explant has been introduced. The present research focuses on the influence of Benzylaminopurine (BAP), Kinetine (KIN), Indolebutyric acid (IBA), Naphthaleneacetic acid (NAA), on rapid *in vitro* propagation of *Aloe vera* plants grown in Bangladesh.

Objectives: Based on the above mentioned context and prospects, the present investigation, therefore, has been carried out with the following objectives:

1. Establishment of *in vitro* regeneration protocol of *Aloe vera*.
2. Assessment of combined hormonal effect for *in vitro* response.
3. To regenerate the plants those are genetically identical to the source material.
4. Acclimatization and cultivation of regenerated plantlets of *Aloe vera*.

CHAPTER II

REVIEW OF LITERATURE

Plant tissue culture forms the backbone of plant biotechnology, which is comprised of micropropagation, induction of somaclones, somatic hybridization, cryopreservation and regeneration of transgenic plants. Plant tissue culture is a technique through which any plant part is cultured on a sterile nutrient medium in controlled light and temperature with the purpose of obtaining growth. The idea of plant tissue culture originated from the cell theory that was formulated by Schwann in 1839. Tissue culture techniques have for decades played a great role in the micropropagation of horticultural and ornamental plants. In fact, the first ever successful plant tissue culture was achieved in horticultural plants (Altman and Ziv, 1997). These techniques have been widely used in disease elimination and vegetative propagation (Husseyg, 1979). Nowadays, it is very common practice all over the world to explore different aspects about *Aloe vera* using this technology. But unfortunately, it is very limited in Bangladesh. However, some related works already performed by different institutes home and abroad have been reviewed and some of the most relevant literatures are cited below.

2.1 Explant

The most researchers proposed the use of shoot tip and apical meristem for micropropagation of aloe (Debiasi *et al*., 2007; Liao *et al*., 2004; Aggarwal and Barna, 2004; Campestrini *et al*., 2006). Other explant, except young stem segments, ceased to grow completely after the discharge of brown substances from the explant. This might be as a result of the fact that juvenile tissues tend to have a greater capacity for restoration (Murashige and Skoog, 1962) or produce less phenols (Roy and Sarkar, 1991).

Baksha *et al*. (2005) studied on micropropagation of *Aloe barbadensis* Mill. through *in vitro* culture of shoot tip explant. Multiple shoot (ten per explant) in *Aloe barbadensis* were obtained from shoot tip explant cultured on MS supplemented with BAP 2.0 mg/L and NAA 0.5 mg/L. About 95% rooting was obtained from micro-shoot cultured on half strength MS supplemented with NAA 0.5 mg/L. Well-developed rooted plantlets were successfully transferred to the soil with 70% survival.

Daneshvar *et al*. (2013) carried out an experiment on the effect of different media on shoot proliferation from the shoot tip of *Aloe vera* L. The results showed that MS media containing 1.5 mg/L KIN along with 0.3 mg/L NAA produced the highest percentage of proliferated shoot. In addition the percentage of proliferated shoot in MS medium containing 2.0 or 2.5 mg/L BAP + 0.15 mg/L NAA was significantly higher than the other treatments.

Hongxing *et al.* (2004) observed relationship between aloin accumulation of *Aloe vera* var. *chinensis* and the callus cultured by the root, stems and leaves as explant. The aloin content in callus was determined by means of HPLC and TLC. The results showed that on the MS medium with 1.0 mg/L NAA + 0.5 mg/L BAP, the differentiation degree of the callus induced from the leaves was in the highest level, meanwhile the callus contained the most aloin. The aloin content was low in the callus from stems. There was no aloin in callus from root. It was also found that on the MS medium with 1.0 mg/L 2,4-D + 0.5 mg/L BAP, the callus differentiation was in low level and without aloin, no matter what organs were used.

Kumawat (2013) focused over *in vitro* regeneration in Ghritkumari (*Aloe barbadensis* Mill.) The explant (micro shoot, leaves and root) of genotypes JA-1 and JA-2 inoculated on MS medium supplemented with standard callus induction protocol (0.5 mg/L KIN + 2.0 mg/L NAA). The cultures were incubated at 25 ± 2^0C under 14:10 photoperiod with a light intensity of 3000 lux. Semi friable, pale yellow callus proliferating from the base of micro shoot explant inoculated on MS medium with 0.25 mg/L KIN + 1.0 mg/L 2,4-D along with different levels of antioxidants (ascorbic acid, activated charcoal and polyvinylpyrrolidone) for *de novo* shoot regeneration.

Lee *et al*. (2011) completed an study on induction and proliferation of adventitious root from *Aloe vera* leaves tissues for *in vitro* production of aloe-emodin. Adventitious root induction was suitable by enrichment of 0.5 mg/L NAA and 0.2 mg/L BAP in Murashige & Skoog (MS) medium. However root proliferation was hindered by accumulation of phenolic compounds in the media that was overcome by pre-washing of the adventitious root with more than 4 g/L of polyvinylphyrollidine (PVP) analogs increasing the survival rate (up to 60 %). Inspection of aloe-emodin contents in various adventitious root grown different basal medium revealed that aloe-emodin accumulation is much higher on B5 medium (133.08 ± 0.12 µg/g) than on MS medium (3.56 ± 0.26 µg/g).

Lobine *et al*. (2015) developed an efficient protocol for a tissue culture strategy towards the rescue of endangered mascarene *Aloes.* The micropropagation and restoration of four endemic threatened Masacrene *Aloes* namely *Aloe lomatophylloides, Aloe macra, Aloe purpurea* and *Aloe tormentorii* were studied. Explant consisting of 2cm long hypocotyls with radicles were cultured on Murashige and Skoog's (MS) basal medium supplemented different plant growth regulators Thidiazuron, Benzyl amino purine and Naphthalene acetic acid (TDZ, BAP, NAA). All explant produced significantly ($p<0.05$) higher number of PLBs on MS containing 0.01 NAA mg/L except for *A. tormentorii* explant whereby more PLBs were obtained in MS with NAA and TDZ. Over 95% of rooted plantlets survived acclimatisation.

Rathore *et al.* (2011) obtained development of tissue culture method for high frequency plantlet regeneration from inflorescence axis derived callus cultures of sweet aloe genotype. Competent callus cultures were established on 0.8% agar gelled MS basal medium supplemented with 6.0 mg/L of 2,4-D and 100 mg/L activated charcoal and additives. The callus cultures were cultured on MS medium containing 1.5 mg/L 2,4-D, 0.25 mg/L Kinetin and additives with 4% carbohydrate source for multiplication and long term maintenance of regenerative callus cultures. The 100% regenerated plantlets were hardened in the greenhouse & stored under an agro net house

Wang *et al*. (2002) used adventitious buds as explant for the propagation of *Aloe vera* tetraploids *in vitro*. The MS medium supplemented with BAP at 1 mg/L was optimum for adventitious bud differentiation and successive culture transfer. MS medium supplemented with IBA at 2 mg/L, NAA at 0.3 mg/L and AC at 0.3 per cent was optimum for successful propagation and differentiation of root.

2.2 Decontamination

Both NaOCl and $HgCl_2$ are oxidizing agents and damage the microorganism by oxidizing the enzymes (Rao, 2008). The ineffectiveness of NaOCl may be due to the reason that it is a mild sterilizing agent (Sirivastava *et al*., 2010). $HgCl_2$ is reported a better sterilizing agent as compared to NaOCl but is more toxic and requires special handling and is difficult to dispose (Maina *et al*., 2010). Apart from its effectiveness, mercuric chloride could be toxic to plant tissues if used in higher concentration (Muna *et al.,* 1999).

Biswas *et al*. (2013) carried out an experiment on micro-propagation of *Aloe indica* L. through shoot tip culture using shoot tip as explant which disinfected with 2% NaOCl

and washing thoroughly with sterile water. After 8 weeks, the best proliferation of average number of shoot per explant was 7.8 for the medium containing of 2 mg/L Benzyladenine with 0.5 mg/L Naphthaleneacetic acid and the lowest average number of shoot per explant was 0.9 for the medium containing of 0.5 mg/L Benzyladenine. Highest average number of root per explant was 5.2 produced in 0.5 mg/L Napthaleneacetic acid concentration. On the other hand, the lowest average number of root per explant was zero with 0.1 mg/L Indole-3-acetic acid concentrations.

de Oliveira and Crocomo (2009) worked on large-scale micropropagation of *Aloe vera.* The effects of two chlorine-based disinfectants were evaluated on the survival of the explant in different treatments in a semisolidified Murashige and Skoog (MS) medium in the presence of 6-benzylaminopurine (6-BAP; 2 mg/L). During 120 days, 136 green apical shoot bearing axillary buds were multiplied four times at 30-day intervals in the same MS medium, reinoculating seven to nine explant per flask each time. The elongation and rooting processes were carried out in the same MS medium without 6-BAP. A total of 40,495 *Aloe vera* microplants were obtained, a yield of 300 microplants per apical bud at a rate of 1:5.3 in every multiplication period of 30 days. From that total, 38,480 *Aloe vera* microplants were successfully acclimatized transferring to 36 and 64 cell polyethylene trays containing proper substrate in two different ex vitro greenhouse conditions.

Hashemabadi and Kaviani (2008) carried out an experiment on rapid micro-propagation of *Aloe vera* L. via shoot multiplication. Explant used for the *in vitro* culture was shoot tip. The shoot tip explant was disinfected with 2% NaOCl and washed thoroughly with sterile water. After 8 weeks, the best proliferation of shoot per explant (9.67) and the best rooting was shown on the medium supplemented with 0.5 mg/L benzyladenine + 0.5 mg/L naphthaleneacetic acid. The rooted plantlets were gradually acclimatized in plastic pots containing a mixture of cocopeat and perlite (1:1) covered with transparent plastic. About 95% of the transplanted plantlets survived.

Monge *et al*. (2008) conducted an experiment on somatic embryogenesis, plant regeneration in Aloe (*Aloe barbadensis* Mill.). For explant disinfection, treatments involved 2, 3, 4, 5, 10 and 15 min sonication, in combination with 4% v/v NaOCl. Explant source and growth regulators were investigated. The highest survival rate (85%) and the lowest contamination (15%) were obtained with 5 min sonication. The highest number of shoot was obtained from embryogenic calluses derived from

zygotic embryos on a medium supplemented with 0.05 mg/L 2,4-D and 2 mg/L BAP. The source of explant has been considered a critical variable for micropropagation in *Aloe vera.* In addition, since not all explant are equal in terms of regenerability, it is likely that different selective pressures would be exerted against different explant. This could result in different frequencies and spectrums of regeneration among plants from different explant.

Sharifkhani *et al*. (2011) studied on an alternative safer sterilization method for explant of *Aloe barbadensis* Mill. This study aims at providing a new method to replace mercuric chloride. In this study sodium hypochlorite (commercial brand Clorox) with some drops of Tween 80 10%, 15%, 20%, 25% and 30% were used. Applying a Kruskal-Walis test (a nonparametric test), revealed that 5% sodium hypochlorite with 20 minutes of hard and constant shaking, gives the highest number (91.7%) of viable and sterilized explant with regeneration potential in Murashige and Skoog medium supplemented with IBA and TDZ and Zeatin.

2.3 Plant growth regulator

Manipulation of the composition and ratio of plant growth regulators (PGRs) is often the primary empirical approach used for optimization of *in vitro* micropropagation methods (Shukla *et al*., 2012).

Aggarwal and Barna (2004) standardized a micropropagation protocol for an elite selection of *Aloe vera* through enhanced axillary branching. MS medium containing 1.0 mg/L BA and 0.2 mg/L IBA gave highest multiplication. Use of 10 mg/L citric acid and liquid medium improved the shoot multiplication. All the micro-shoot produced rooted plantlets within 15 days of culture on hormone free agar medium. Use of liquid medium, during rooting stage, decreased frequency of rooting. The plants were successfully transferred to the soil and were morphologically similar to mother plants.

Bhandari *et al*. (2010) studied over *in vitro* propagation of *Aloe vera.* Its growth was found well in different concentrations of BA (6-Benzylaminopurine), IBA (3-Indol butyric acid), KIN (Kinetin) and adenine sulphate. Both BA and KIN were found to give the indications of shoot proliferation after 2 weeks of incubation. It was found that BA (1.0 mg/L) gave better shoot proliferation (3.3 ± 1.1) than Kn. Hundred percent cultures showed shoot proliferation on BA containing medium.

Budhiani (2001) demonstrated an experiment on *in vitro* propagation of *Aloe vera*- a plant with medicinal properties. On that experiment, the best initiation and

multiplication of shoot obtained on MS medium with 0.2 mg/L BAP + 0.002 mg/L NAA (1.50±1.29 shoot) and 2.0 mg/L BAP + 0.002 mg/L NAA (1.8±1.09 shoot), respectively. Highest number of shoot was shown on the 4th subculture, produced 12.67±0.52 shoot.

Chaudhuri and Mukundan (2001) cultured shoot tips of *Aloe vera* on full strength MS medium containing 3 per cent sucrose and supplemented with adenine sulfate (Ads), benzyladenine (BA), IAA and IBA alone or in combinations. Leavesy shoot differentiated in almost all the treatments. 60 days after culture, 2-3 cm shoot were sub-cultured for 4 weeks on half-strength MS medium supplemented with 1.0 mg/L IAA. The formation of multiple shoot *in vitro* was due to the presence of cytokinin and auxin. However, the presence of only auxin or only cytokinin in the culture medium resulted in root or callus formation. The optimal medium for maximum shoot formation was full strength MS medium + 10 mg/L BA + 160 mg/L AS + 0.1 mg/L IBA. Leavesy shoot cultured on half-strength MS medium + 1 mg/L IAA produced as many as 20 shoot/explant. The fresh gel exudate from both the control plants and tissue culture derived acclimatized plants contained about 1 per cent dry matter. The average level of soluble solids was 0.6 - 0.7 per cent and fresh weight and fiber content was 0.075 per cent. The gels from both types of plants were similar and mainly comprised of reducing sugars (mannose and glucose in 3:1 ratio).

Choudhary *et al.* (2011) observed profuse callus induction in shoot tip of *Aloe vera* on MS medium with 0.5 mg/L KIN and 0.5 mg/L 2,4- D and shoot formation with 1.0 mg/L BAP and 0.5 mg/L NAA. Many plants that are rich in polyphenolic compounds inhibit the primary cultures, such compound turned the media brown when the explant were cultured due to the oxidation of polyphenolic compound from explant. *Aloe vera* by such problems, the explant transferred on fresh medium every two weeks.

Chukwujekwu (2001) conducted an experiment on micropropagation and acclimatization of *Aloe polyphylla* and *Platycerium bifurcatum*. Shoot cultures of *Aloe polyphylla* were initiated from young shoot explant of *in vitro* grown plants. The basal medium was MS medium (Murashige and Skoog, 1962), supplemented with 100 mg/L myo-inositol, and 30 g/L sucrose. Agar (0.8 %) was used as the gelling agent. Different cytokinins, singly or in combination with auxins (IBA and NAA), were tested for shoot proliferation activity. All the cytokinins tested (kinetin, zeatin, iP, and BA) gave a good shoot proliferation response. The optimal concentrations for shoot proliferation of each of the cytokinins tested were: zeatin (0.5 mg/L), kinetin

(1.5 mg/L), iP (1.0 mg/L) and BA (1.5 mg/L). In combination with auxins, the optimal combinations were kinetin/NAA (2.0/0.1 mg/L), kinetin/BA (1.5/1.0 mg/L), zeatin/BA (1.0/0.5 mg/L), zeatin/NAA (1.0/1.0 mg/L), BA/IBA (1.0/1.0 mg/L), BA/NAA (1.5/0.1 mg/L). Although it gave the highest number of shoot per explant, BA was responsible for hyperhydricity. Temperature and sucrose also influenced shoot proliferation. The optimal temperature was 25°C, while 30 g/L was the optimal concentration of sucrose for shoot proliferation. Plants rooted well in plant growth regulator free MS medium. Amongst the potting mixtures tested, soil: sand: vermiculite (1:1:1 v/v) was the best with 98 % plantlet survival.

Haque and Ghosh (2013) conducted an experiment on high frequency microcloning of *Aloe vera* and their true-to-type conformity by molecular cytogenetic assessment of two years old field growing regenerated plants. During experimenting nodal portion of rhizomatous stem of *Aloe vera* were cultured on Murashige and Skoog (MS) medium supplemented with various cytokinin and *Aloe vera* leaves gel (AvG) as organic supplement. Number of proliferated shoot per explant was increased along with the regeneration cycles and on MS medium supplemented with 2.5 mg/L 6-benzylaminopurine and 10.0% (v/v) AvG, only 17.8 ± 0.35 shoot per explant were induced on 1st regeneration cycle whereas on 3rd regeneration cycle these number increase to 38.5 ± 0.44 shoot per explant on the same medium composition. After transfer of individual excised shoot to a one third strength MS medium containing 20.0% (v/v) AvG, all the shoot formed whole plantlets with maximum number (9.6 ±0.29) of root per shoot. 95.0% of the regenerated plantlets survived on poly-green house.

Hashemabadi and Kaviani (2010) studied on *in vitro* proliferation of an important medicinal plant *Aloe vera*. In this study, shoot tips of *Aloe vera* L., was cultured in Murashige and Skoog (MS). Explant were cultured on medium containing different concentrations of benzyladenine (BA), Indol-3-butyric acid (IBA) and α-naphthalene acetic acid (NAA). The best proliferation of shoot per explant (9.67) and rooting were shown on medium supplemented with 0.5 mg/L BA + 0.5 mg/L NAA. The largest number of root was obtained on medium supplemented with 0 mg/L IBA + 1 mg/L NAA (9.71). The longest (8.75 cm) and thickest (4.3 cm) root were achieved on medium supplemented with 1 mg/L IBA + 1 mg/L NAA. Minimum microshoot were obtained in control plants. In all stages of this experiment, regenerated plants were

transferred to cocopeat and perlite (1:1) after hardening and they showed 100% of survival.

Nayanakantha *et al*. (2010) developed an efficient micropropagation protocol under an experiment named improved culture medium for micropropagation of *Aloe vera* L using lateral shoot explant of *Aloe vera*. Both shoot induction and elongation were better on MS medium supplemented with 4mg/L BAP + 0.2mg/L NAA + 1g/L PVP. All cultures showed shoot regeneration in this medium with 16 shoot/ explant. More adventitious buds (21.5 shoot/explant) developed on MS medium supplemented with 4mg/L BAP + 0.2mg/L NAA + 1g/L PVP + 10mg/L citric acid + 0.5g/L activated charcoal. Further elongation and rooting of micro-shoot were obtained when sub-cultured on to MS basal medium containing 0.5g/L activated charcoal and 100% of the survival of rooted plantlets was observed after acclimatization.

Patel and Sail (2012) observed highest callus induction in the media supplemented with 0.5 mg/L NAA. The callus was subcultured in the 0.5 mg/L NAA and the quantity of callus increased within 6 days.

Tanabe and Horiuchi (2006) carried out an experiment on *ex vitro* autotrophic culture of *Aloe barbadensis* Mill.. Aloe plants can be grown autotrophically in a maintenance-free, sterile, *ex vitro* environment. Excel, 15-5-15 plus minors fertilizer formulation and Plant Preservative Medium (PPM) biocide was used as a very economical basal medium. In vitro micropropagated plantlets were regenerated in excel media with 2 g /L sucrose and 2.2 µM 6-Benzylamino purine (BA) and used to initiate *ex vitro* cultures in open vessels. *Ex vitro* media loss due to evaporation was reduced by sealing the culture vessel around the plantlets with either parafi lm or aluminum foil and by adjusting gellan gum levels. Parafi lm and higher levels of gellan gum signifi cantly reduced ex vitro media loss but higher gellan gum levels (6 g /L or 8 g /L) also reduced plant weight gains compared to plants grown in 4 g /L gellan gum for 25 weeks after transplanting.

Ujjwala (2006) obtained higher regeneration frequency (80%) of shoot from shoot tip segment on MS medium fortified with 4 mg/L BA and 1 mg/L NAA. The regenerated shoot rooted best on medium containing 1 mg/L IBA with 0.5% activated charcoal. Regenerated plantlets with well developed shoot and root were hardened and successfully transferred to soil.

Wu and Xie (2002) studied the effect of hormones, sugar and activated charcoal (AC) on shoot propagation of *Aloe vera*. Root formation was studied using tender or lateral

buds explant. The best medium for shoot propagation was MS media + 5 mg/L BA + 0.1 mg/L NAA + 30 gm/L sugar + 0.5 per cent activated charcoal with a propagation coefficient of 51-54 after being cultured for 20-25 days. The suitable medium for root formation was MS media + 0.8 mg/L NAA + 30 gm sugar/L + 0.3 per cent activated charcoal. The number of root was 4-5 and the height of shoot was 5-6 cm after having cultured for 15 days.

Yadav (2008) observed profuse callus induction in adventitious bud of *Aloe barbadensis* Mill., cultured on MS medium supplemented with 0.5 mg/L KIN + 1.5 mg/L NAA. Mmaximum shoot induction in microshoot explant of *Aloe vera* when, cultures incubated at 14:10 hours photoperiod followed by 12:12 hours photoperiod. The callus also remained potent during subculture.

Zakia *et al. (*2013) accomplished standardization of micropropgation techniques for *Aloe vera*: Shoot tip was used as an explant for *in vitro* regeneration of *Aloe vera*. Explant were disinfested with the use of 0.1% mercuric chloride and 0.5% sodium hypochlorite, and washed thoroughly with autoclaved distilled water. Solid MS medium was used with additionof different concentrations of 6-benzyl aminopurine and α-naphthalene acitic acid. After 7 weeks of inoculation, greatest number of shoot (11.18) and highest shoot length (12.15cm) were found in MS medium supplemented with 0.5 mg l-1 6- benzylaminopurine (BAP) along with same concentration of α-naphthalene acitic acid (NAA). Best rooting (84.67%) was found in medium supplemented with 1.5 mg/L of indole butyric acid (IBA). The rooted explant were thengradually acclimatized and shifted to green house.

Gurjar (2009) observed maximum *de novo* shoot regeneration in callus cultures of *Aloe vera* supplemented with 0.25 mg/L KIN + 1 mg /L 2,4-D and incubated at 16:8 hours photoperiod followed by 14:10 hours photoperiod. The *de novo* shoot regeneration from callus culture was completely inhibited at 8:16 hours photoperiod.

Jakhar *et al.* (2012) observed profuse callus induction in shoot apex explant of *Aloe vera* when cultured on MS medium supplemented with 0.5 mg/L KIN + 2.0 mg/L NAA and incubated at 25 ± 2^0C with photoperiod of 14:10 hours.

Kawai *et al.* (2006) observed callus induction in the tissue of *Aloe arborescens* on MS medium supplemented with 3% sucrose, 0.1- 0.5 μM/L KIN and 10-15 μM/L NAA. Callus formation occurred at incidence of 20-32% (mean 39%) when the MS basal agar medium was supplemented with 3% glucose and 10% Alpha modification of Eagles medium instead of 3% sucrose, callus formation was promoted.

Saggoo and Kaur (2010) reported shoot proliferation in callus induced from shoot disc of *Aloe vera* on MS medium supplemented with 1.0 mg/L 2.4-D + 0.2 mg/L kinetin when, cultured on MS + 0.2 mg/L BA + 0.2 mg/L IBA. For rooting MS + 0.3 mg/L NAA gives best result. Complete regenerated plantlets from callus were hardened, acclimatized and transferred in garden soil. They further suggested that regeneration of plants from callus may help to induce variability in the *Aloe* germplasm for improvement.

Singh, B and Sood, N (2009) moved with significance of explant preparation and sizing in *Aloe vera* L. A highly efficient method for in vitro multiple shoot induction. The stem nodal explant and shoot tips were cultured on modified Murashige and Skoog's medium (1962) supplemented with different concentrations of 6-benzylaminopurine (BA), kinetin (KIN), indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) and anaphthaleneacetic acid (NAA) either singly or in combination. The best media composition was found to be MS medium supplemented with IAA (11.42 mM), IBA (9.8 mM) and BA (8.88 mM). The explantwere divided into 2 sets, with and without ensheathing leaves base. Explant sizing, pruning and retention of mother tissue was highly significant in induction of multiple shoot and root. The stem nodal explant with leaves base performed much better than those without such covering. A very high number of shoot and root grew from these explant. The rooted plantlets were successfully acclimatized and transferred to the green house conditions and finally to field conditions.

2.4 Hardening

The produced plants are very soft to face ambient environmental condition (Bhojwani and Razdan, 1992).

Ahmed *et al.* (2007) conducted an experiment on development of rapid micropropagation method of *Aloe vera* L. using shoot tip explant. Shoot proliferation was found best in MS medium containing BA 2.0 mg/L, KIN 0.5 mg/L and NAA 0.2 mg/L. Maximum 98.96% shoot were proliferated in this media composition. This media composition is best comparing to other treatment used in this study. Highest shoot number per explant was also achieved in the same medium within 5 weeks. In case of adventitious rooting, MS medium containing NAA 0.2 mg/L and NAA 0.5 mg/L was found to be the best. Maximum 80.25% rooting and highest number of root per culture (6.71) was obtained in this media composition. After transplanting the 20 days old rooted shoot into mixture of garden soil, compost and sand (2:1:1), 80% of

survivability after 5 weeks was an achieved. Regenerated plants after acclimatization were transferred to soil and they showed 82% survival.

Dwivedi *et al*. (2014) standardized a protocol for micropropagation of *Aloe vera* L. axillary shoot of *Aloe vera* (Indian Aloe) produced new plants and root simultaneously when cultured in MS medium supplemented with BAP 1.5 mg/L. Each explant produced on average 14 shoot and root simultaneously within 8 weeks. Explant multiplication could be continued even after a year by transferring each divided shoot explant to the same medium. Regenerated plantlets could be successfully transferred to the soil where they grew well within 4-6 weeks with 83% survival.

Gholamreza *et al.* (2013) worked on *in vitro* micropropagation of *Aloe vera.* They observed the effect of explant of shoot tip (with and without sheath type A and B) in *Aloe vera.* These explant were cultured on MS, B5 and SH media supplemented with different combination of NAA with BAP and Kn for shoot induction. The highest shoot proliferation response obtained successfully by using MS medium containing 4 mg/L BA. The optimal rooting response was observed on B5 medium supplemented with 2 mg/L NAA, on which 100% of the regenerated shoot developed root with an average of 7.8 root per shoot within 3 weeks. The plantlets were acclimatized and transferred to greenhouse with 95% success.

Hasanuzzaman *et al*. (2008) accomplished an experiment on plant characteristics, growth and leaves yield of *Aloe vera* as affected by organic manure in pot culture. There were 8 different treatments viz., T1 = 100% soil (control), T2= 50% cowdung + 50% soil, T3= 25% cowdung + 75% soil, T4= 10% cowdung + 90% soil + urea, T5= 10% cowdung + 90% soil, T6=5% cowdung + 95% soil + urea, T7= 5% cowdung + 95% soil, T8=soil + urea. It was observed that the plant produced highest number of leaves and maximum leaves weight, maximum leaves breadth, total leaves area as well as length and breadth of largest leaves with application of 50% cowdung + 50% soil (T2). Different plant characters such as weight of tiller, stem and root as well as root length was also found to be highest with T2 treatment over control (100% soil).

CHAPTER III
MATERIALS AND METHODS

3.1 Time and location of the experiment

The present research was carried out in Biotecnnology Laboratory of the Department of Biotechnology, Sher-e-Bangla Agricultural University, Sher-e-Bangla Nagar, Dhaka-1207 from the period of september 2013 to July 2014.

3.2 Experimental materials

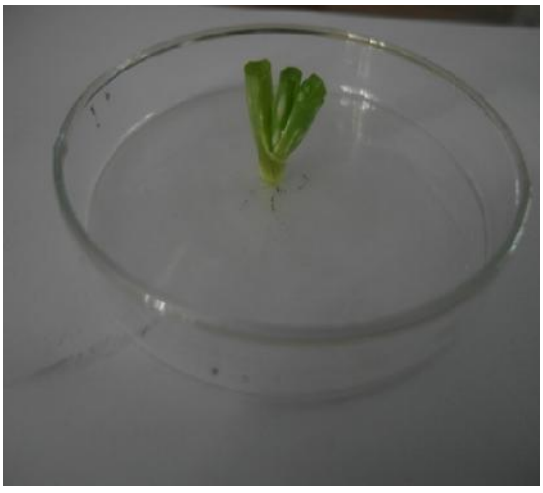
3.2.1 Source of material

The planting materials of *Aloe vera* were collected from Agargaon Nursery, Sher-e-Bangla Nager, Dhaka-1207.

3.2.2 Plant materials

Fresh, healthy and disease free lateral shoot (suckers) of *Aloe vera* were harvested in a beaker filled with water. The explant were washed thoroughly with running tap water for removing soil from root. Shoot with young leaves were collected from the elite plants showing good biomass yield (Plate 1A). The extra leaves were removed and shoot were trimmed to size of 2-3 cm for further work (Plate 1B).

1A



1B

Plate 1. Plant material of *Aloe vera* for micropropagation

3.2.3 Instruments

Metal instruments *viz*., forceps, scalpels, needless, spatulas and aluminum foils were sterilized in an autoclave at a temperature of 121^0C for 20 minutes at 1.06 kg/cm^2 (15 PSI) pressure.

3.2.4 Glass ware

The Borosil glassware were used for all the experiments. Oven dried (250^0C) Erlesn meyer flasks, culture bottles, flat bottom flasks, pipettes, petridishes, beaker and measuring cylinders (25 ml, 50 ml, 100 ml, 500 ml and 1000 ml) were used for media preparation**.** The glassware were first rinsed with the liquid detergent (Trix) and washed thoroughly with tap water until the detergent was removed completely. Finally they were rinsed with distilled water and sterilized in oven at 160-180^0C for 3-4 hours.

3.2.5 Culture medium

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. Explant were inoculated onto media composed of basal MS (Murashige and Skoog, 1962) medium supplemented with the plant growth regulators. Composition of MS media have been shown in appendix I. Hormones were added separately to different media according to the requirements. To do so, stock solutions of hormones were prepared ahead of media preparation and stored at 4^0C temperature.

1. BA (0.5, 1.0, 1.5 and 2.0 mg/L) alone or in combination with KIN (0.25, 0.5, 0.75 and 1.0 mg/L), NAA (0.25, 0.5, 0.75 and 1.0 mg/L), and IBA (0.25, 0.5, 0.75 and 1.0 mg/L) were used for shoot proliferation.

2. NAA (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/L) and IBA (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/L) were applied separately for root formation.

3. Sucrose (3%) was used as carbon source and media were solidified with agar-agar (0.8%).
4. The pH was adjusted to pH 5.8 prior to autoclaving at a temperature of 121^0C for 20 minutes at 1.06 kg/cm^2 (15 PSI) pressure.

3.3 Preparation of the stock solution of hormones

To prepare these hormonal supplements, they were dissolved in proper solvent as shown against each of them below. Generally, cytokinins were dissolved in few drops of acidic solutions (1N HCl) and Auxins were dissolved in few drops of basic solutions (1N NaOH).

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

In present experiment, the stock solution of hormones were prepared by following procedure. 100 mg of solid hormone was placed in a small beaker and then dissolved in 10 ml of 70% ethyl alcohol or 1 (N) NaOH solvent. 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. (Growth regulators were purchased from Sigma, USA).

3.4 Preparation of culture 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 gm of MS media and 30 gm 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 gm agar was added to the mixture and heated for 10 minutes in an electric oven for melting of agar.

3.5 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 30 minutes at 1.06 kg/cm^2 (15 PSI) pressure. After autoclaving the media were stored in at 25±2 °C for several hours to make it ready for inoculation with explant.

3.6 Preparation of explant

The trimmed shoot tips were washed thoroughly under running tap water and then with autoclaved distilled water for several times. Subsequently the explant were transferred to laminar airflow cabinet and kept in a 250 ml sterilized beaker. The beaker with explant was constantly shaken during sterilization. They were treated

with 70% ethanol for 1-2 minute and rinsed with autoclave distilled water for 3-4 times. After treating with 70% ethanol, the explant 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. Then explant were washed 3-4 times with autoclaved distilled water to make the material free from chemical and ready for inoculation in culture media.

3.7 Inoculation of culture

The sterilized explant 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, instruments in use were placed in a beaker containing 70 % ethanol and were flamed repeatedly using a spirit burner. The worker hands and forearms were washed thoroughly with soap and water and repeatedly sprayed with 70% alcohol during the period of work. The mouth of culture vial was flamed before and after positioning of the explant on the medium.

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

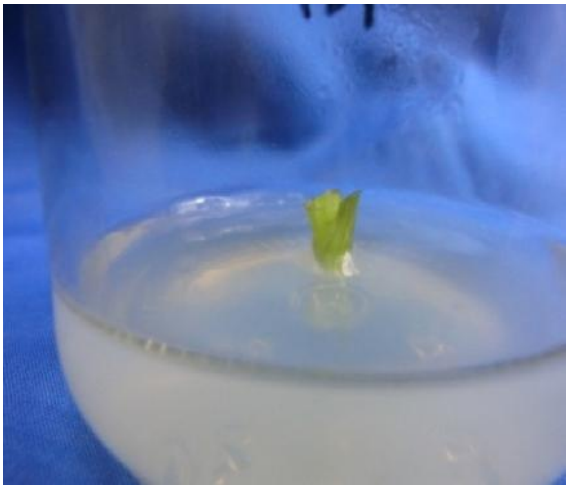


Plate 2. Inoculation of culture with the explant of *Aloe vera* L.

3.8 Incubation

The bottles were kept to the culture racks and allowed to grow in controlled environment (Plate 3). The cultures were maintained at 25±2 °C with light intensity varied from 2000–3000 lux (23 W white bulbs). White fluorescent lamps were used for growth of the culture. The photoperiod was generally 14 hours light and 10 hours dark having 70% relative humidity (RH).

Plate 3. Incubation of inoculated culture vial.

3.9 Maintenance of proliferating shoot

The explant were cultured on MS nutrient medium supplemented with different concentration of BAP alone or in combination of KIN, NAA and IBA. After successful shoot proliferation, subculture was done with newly formed shoot. Shoot ware excised in aseptic condition with the 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 shoot were made throughout the entire culture period. Data recording was started after 2 weeks from inoculation.

3.10 Root formation of regenerated shoot

Newly formed shoot with adequte length were excised individually from the culture vial and transferred to rooting media. Two types of growth regulators (IBA and NAA) were used separtely in different concentration (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/L) along with MS media. The observations on development pattern of root were made throughout the entire culture period. Data were recorded from 3rd week of inoculation.

3.11 Acclimatization

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

Step-1: After 35 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 root 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 root, 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 (2 days interval).

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

3.12 Data recording

The observation on development pattern of shoot and root were made throughout the culture period. Five replicates each of them containing 4 bottles (single shoot per culture bottle) were used per treatment. Data were recorded after 3, 6 and 9 weeks of culture, starting from day of inoculation on culture media in case of shoot proliferation. In event of root formation, it was done every week starting from third week to fifth week of culture. The following observations were recorded in cases of shoot and root formation under *in vitro* condition.

1. Percent of explant showing shoot induction
2. Days to shoot induction
3. Number of shoots per explant
4. Average length of shoot (cm)
5. Number of leaves per explant
6. Percent of explant showing root induction
7. Days to root induction
8. Number of roots per explant
9. Average length of root (cm)
10. Diameter of root (mm)

3.12.1 Calculation of percent of shoot and root induction from culture:

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

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

The percentage of root induction was calculated as:

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

3.12.2 Calculation of days to shoot and root induction

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

3.12.3 Calculation of number of shoot and root per explant

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

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

3.12.4 Calculation of number of leaves

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

3.12.5 Calculation of shoot and root length (cm)

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

3.12.6 Calculation of diameter of root per explant

Root diameter was measured in centimeter (mm) with a screw micrometer by using the following formula,

Total reading = Linear scale reading (mm) + Circular scale reading (mm)

Then the mean was calculated.

3.12.7 Calculation of percent of plant establishment:

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

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

3.12.8 Calculation of percent increase over control (%)

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

$$\text{Percent increase over control} = \frac{X_1 - X_2}{X_2} \times 100$$

Where, X_1 = the mean of treated explant,

X_2 = the mean of untreated explant

3.13 Statistical analysis

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

CHAPTER IV
RESULTS AND DISCUSSION

Three separate experiments were performed for the rapid micropropagation of the medicinally important herb *Aloe vera*. The overall objective of the present study has been to develop a system for the mass propagation of *Aloe vera*. The results of these experiments have been presented and discussed in this chapter with Plates (4-13), Figures (1-15) and Tables (1-15). Analyses of variance in respect of all the parameters have been presented in Appendices (II-XXXV).

4.1 Experiment 1. Multiple shoot proliferation in *Aloe vera* L.

This experiment was conducted under laboratory condition to evaluate the effect of different plant growth regulators on multi shoot proliferation. Manipulating the relative ratio of auxin to cytokinin has been successfully used in the current investigation. The response of explant to different plant growth regulators singly or in combination varied significantly. The results are presented separately under different headings below.

4.1.1 Effect of BAP on multiple shoot proliferation

The results of the effect of different concentrations of BAP have been presented under following headings with Figures 1-5 and Plate 4.

4.1.1.1 Percent of explant showing shoot induction

There was significant variation on percent of explant showing shoot induction at different concentration of BAP. The highest percentage (60%) of shoot induction was induced in treatment 0.5 mg/L BAP and the lowest percentage (35%) was induced in hormone free media (Figure 1). Baksha *et al.* (2005) reported the maximum 30% shoot induction in 2.0 mg/L BAP and 20 % in 0.5 mg/L BAP. There was a declining trend of shooting in *Aloe vera* with increasing the concentration of BAP. This decline

is also supported by Hashemabadi and Kaviani (2008). Jaramillo *et al.* (2008) reported that presence of BAP in the culture medium is necessary for shoot regeneration although higher concentration reduced the shoot regeneration frequency.

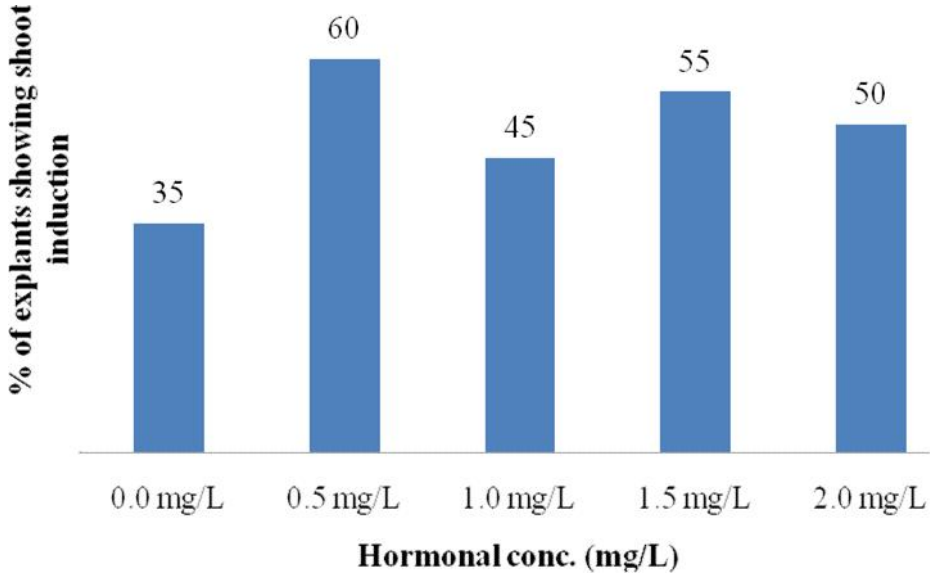


Figure 1: Effect of BAP on percent of explant showing shoot induction in *Aloe vera* L.

4.1.1.2 Days to shoot induction

Significant variations were observed among different concentration of BAP on days to shoot induction. The maximum days to shoot induction were recorded in control (41 days) and 0.5 mg/L required minimum 23 days (Figure 2).

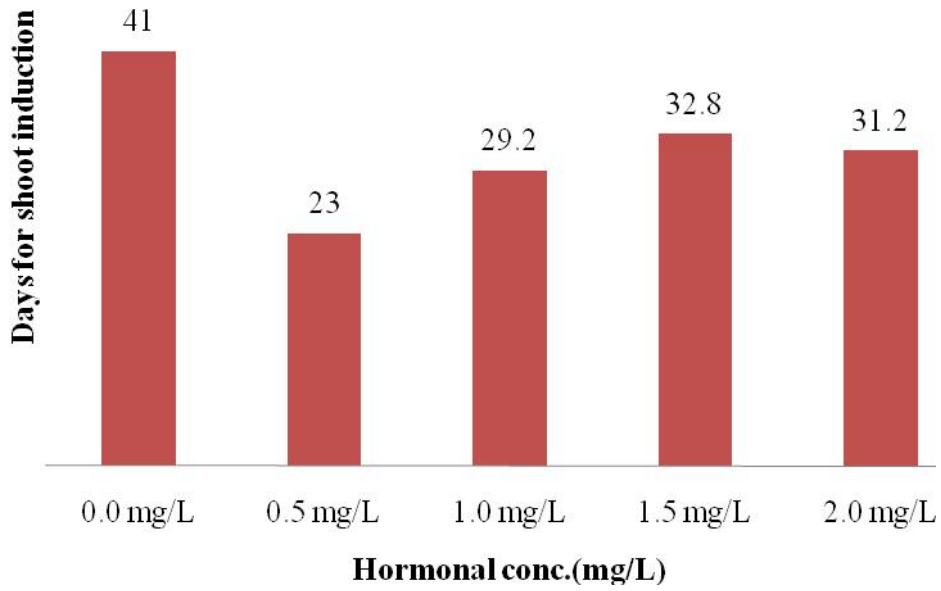


Figure 2: Effect of BAP on days to shoot induction in *Aloe vera* L.

4.1.1.3 Number of shoots per explant

There was significant influence of different concentrations of BAP on the number of shoots per explant. Data were recorded after 3, 6 and 9 weeks of culture on MS media. The results have been presented in Figure 3 and Plate 4. There were no significant variations at 3 WAI among different concentrations of BAP. However, 0.5 mg/L BAP gave the highest number of shoots (2.4 and 4.2 at 6 WAI and 9 WAI respectively) whereas the lowest number of shoots (1.2 and 1.4 at 6 WAI and 9 WAI respectively) was found with hormone free media. Variations in shoot proliferation due to BAP conc. were also reported by Bhandari *et al*. (2010) and Gantait *et al.* (2010). Baksha *et al.* (2005) noticed 3.2 shoots per explant in media supplemented with 2.0 mg/L BAP.

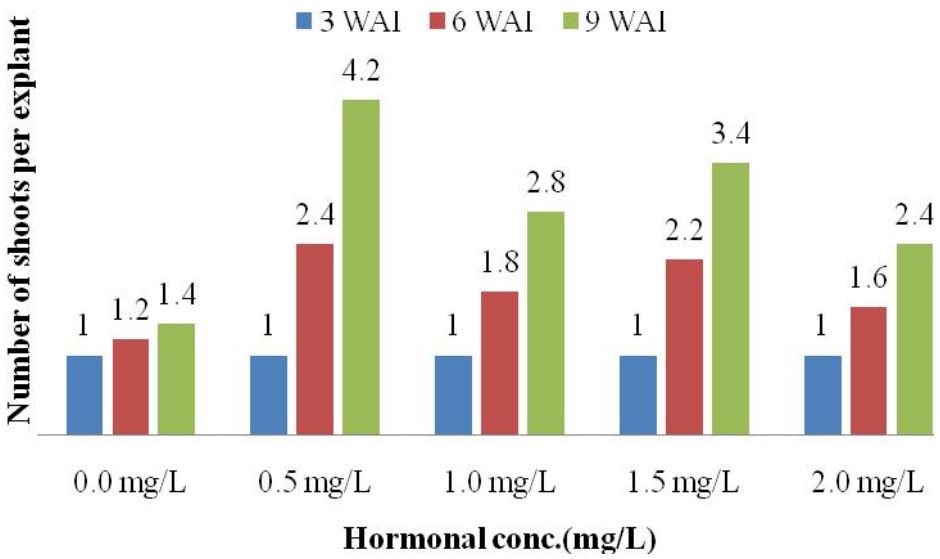
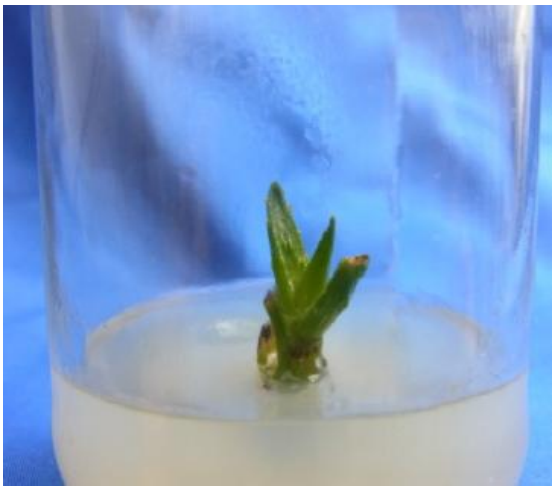
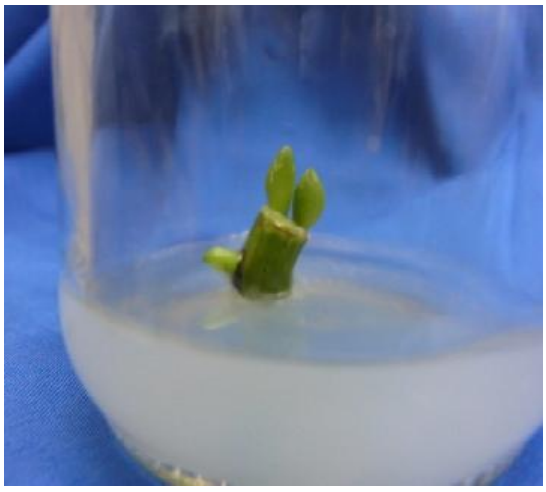


Figure 3: Effect of BAP on the number of shoots per explant in *Aloe vera* L.

Qu *et al.* (2000) reported that presence of high cytokinin level in the medium cause's cytogenetic instability; thus unsuitable for clonal propagation. Moreover, most of the shoots so formed were stunted.





4A **4B**

4C

Plate 4: Shoot proliferation of *Aloe vera* on MS media supplemented with 0.5 mg/L BAP. (A) after 3 weeks, (B) after 6 weeks and (C) after 9 weeks of inoculation

4.1.1.4 Number of leaves per explant

With different concentrations of BAP, significant variation was found on the number of leaves. The highest 7.6 leaves were recorded with 0.5 mg/L BAP and the lowest 2.6 in case of lack of hormone (Figure 4).

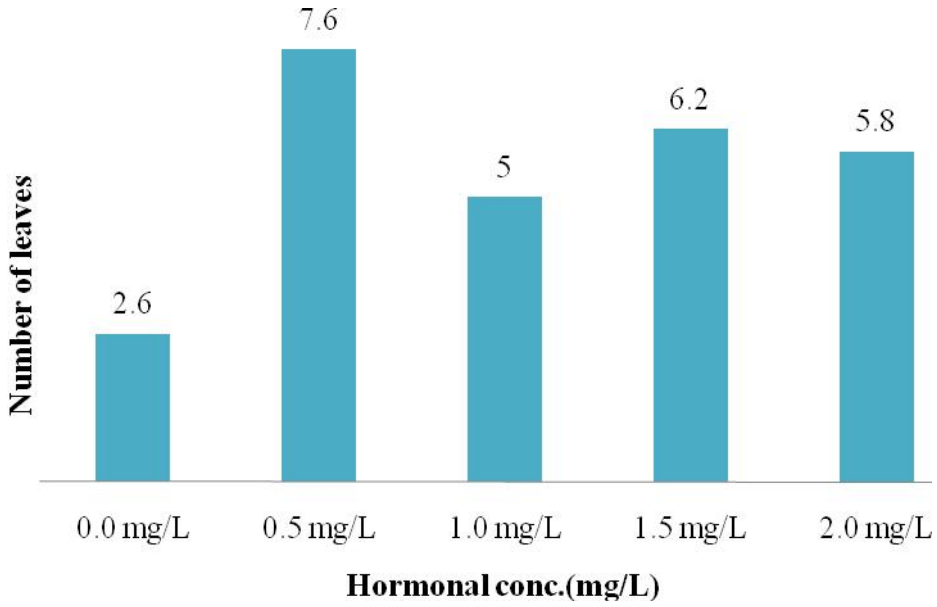


Figure 4: Effect of BAP on number of leaves per explant in *Aloe vera* L. at 9 WAI

4.1.1.5 Average length of shoot (cm)

The results of average length of shoot have been presented in Figure 5. The highest average length of shoot (4.46 cm) was noticed from the 1.0 mg/L BAP which was statistically similar with 1.5 mg/L BAP (4.24 cm) and statistically different from rest of the treatments whereas the minimum 3.30 cm was found in control. Baksha *et al.* (2005) noticed 2.5 cm length of shoot in 4.0 mg/L BAP.

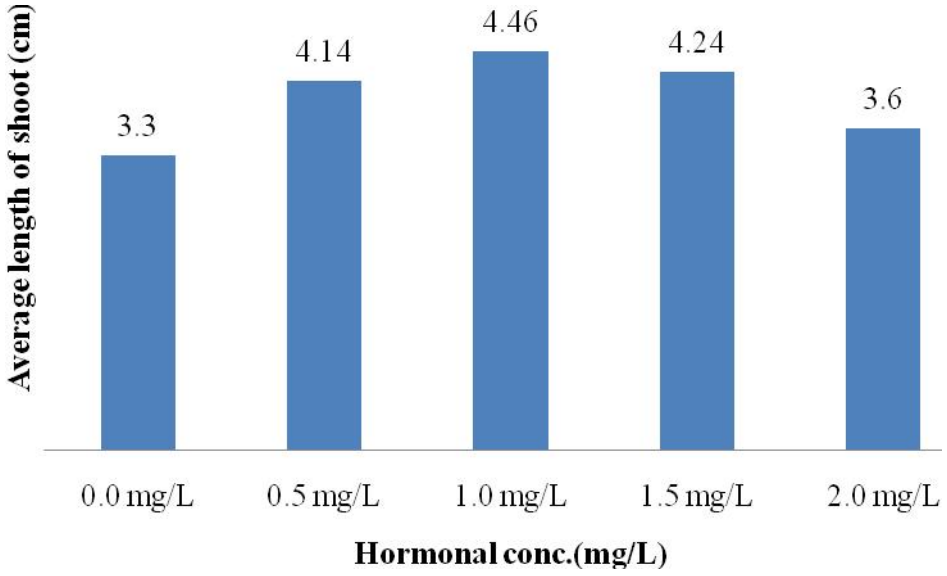


Figure 5: Effect of BAP on average length of shoot (cm) in *Aloe vera* L. at 9 WAI

This decline in the shoot length of *Aloe vera* might be due to the inhibitory effect of BAP, which provoke a little suppression of plant growth and activity of some proteolytic enzymes (Petkova *et al.*, 2003).

4.1.2 The combine effect of BAP + KIN on multiple shoot proliferation

The result of the combined effect of different concentrations of BAP + KIN have been presented under following headings with Tables 1-3, Figure 6 and Plate 5.

4.1.2.1 Percent of explant showing shoot induction

There was significant variation of BAP + KIN concentration on percent of explant showing shoot induction. The highest percentage (75%) of shoot induction was

induced in treatment 1.0 mg/L BAP + 0.75 mg/L KIN and the lowest percentage (35%) was induced in hormone free media (Table 1).

Table 1. Combined effect of BAP and KIN on percent of explant showing shoot induction

BAP (mg/L)	KIN (mg/L)	Number of explant inoculated	Number of explant initiated shoot	Percent of explant showing shoot induction
MS-Control		20	7	35
0.5	0.25	20	10	50
	0.5	20	13	65
	0.75	20	12	60
	1.0	20	13	65
1.0	0.25	20	13	65
	0.5	20	14	70
	0.75	20	15	75
	1.0	20	14	70
1.5	0.25	20	11	55
	0.5	20	13	65
	0.75	20	11	55
	1.0	20	12	60
2.0	0.25	20	9	45
	0.5	20	10	50
	0.75	20	11	55
	1.0	20	10	50

4.1.2.2 Days to shoot induction

Variations were observed among different concentrations of BAP + KIN on days to shoot induction. The highest number of days to shoot induction was recorded in control (41 days) and 1.0 mg/L BAP + 0.75 mg/L KIN required lowest (18.8 days) (Table 2).

Table 2. Combined effect of BAP and KIN on shoot induction potentiality

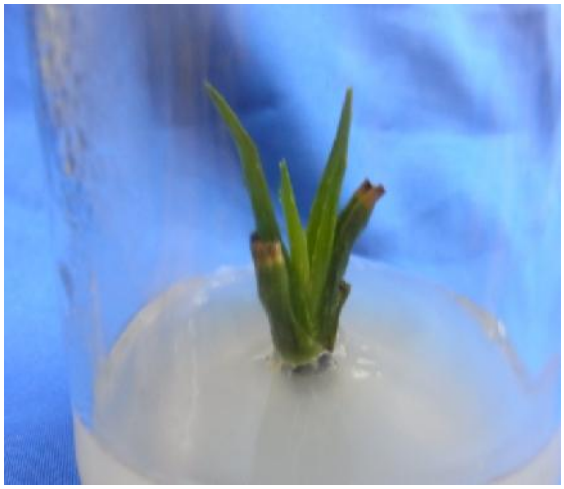
BAP (mg/L)	KIN (mg/L)	Shoot induction potentiality				
		Days for shoot induction	Number of shoots per explant			% increase over control
			3 WAI	6 WAI	9	

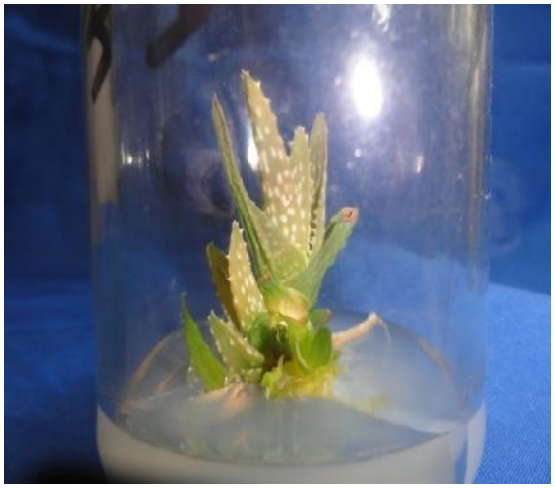
					WAI	**(9 WAI)**
MS-Control		41.0 a	1	1.2 f	1.4 i	-
0.5	0.25	23.0 gh	1	1.4 f	3.4 fg	142.85
	0.5	23.8 fg	1	1.6 ef	4.0 ef	185.71
	0.75	24.8 ef	1	2.4 cde	5.2 cde	271.43
	1.0	21.6 i	1	2.0 def	4.6 def	228.57
1.0	0.25	26.6 cd	1	2.6 bcd	5.0 cde	257.14
	0.5	21.6 hi	1	3.4 ab	5.8 cd	314.28
	0.75	18.8 j	1	3.8 a	7.4 a	428.57
	1.0	20.2 i	1	3.6 a	4.0 ef	185.71
1.5	0.25	27.2 bc	1	3.2 abc	6.0 bc	328.57
	0.5	28.0 b	1	3.4 ab	7.0 ab	400.00
	0.75	24.8 ef	1	3.0 abc	5.6 cd	300.00
	1.0	23.2 g	1	3.4 ab	4.2 ef	200.00
2.0	0.25	24.4 efg	1	3.0 abc	5.0 cde	257.14
	0.5	23.0 gh	1	1.6 ef	2.6 gh	132.65
	0.75	20.8 i	1	1.6 ef	3.6 fg	157.14
	1.0	25.4 de	1	1.4 f	1.8 hi	56.00
CV%		4.25	-	26.33	18.95	-
LSD value		1.320	-	0.8324	1.078	-

*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.

4.1.2.3 Number of shoots per explant

Data were recorded after 3, 6 and 9 weeks of culture on MS media. There was significant influence of different concentrations of BAP + KIN on the number of shoots per explant after 3rd weeks of inoculation. The results have been presented in Table 2 and Plate 5. There were no significant variations at 3 WAI among different concentrations of BAP + KIN. But 1.0 mg/L BAP + 0.75 mg/L KIN gave the highest number of shoots (3.8 and 7.4 at 6 WAI and 9 WAI respectively) whereas the lowest number of shoots (1.2 and 1.4 at 6 WAI and 9 WAI respectively) was found with hormone free media.





5A **5B**

5C

Plate 5: Shoot proliferation of *Aloe vera* on MS media supplemented with 1.0 mg/L BAP + 0.75 mg/L KIN. (A) after 3 weeks, (B) after 6 weeks and (C) after 9 weeks of inoculation

4.1.2.4 Number of leaves per explant

The Number of leaves per explant was significantly different due to the different concentrations of BAP + KIN supplemented. The results have been presented in Table

3. The highest number of leaves per explant (13.4) was noticed from 1.0 mg/L BAP + 0.75 mg/L KIN statistically similar with 1.5 mg/L BAP + 0.5 mg/L KIN (12.2), whereas the lowest was 2.6 in control.

Table 3. Combined effect of BAP and KIN on the number of leaves per explant in *Aloe vera* L. at 9 WAI

BAP (mg/L)	KIN (mg/L)	No. of leaves per explant	% increase over control
MS-Control		2.6 i	-
0.5	0.25	7.6 efg	192.31
	0.5	10.4 c	300.00
	0.75	8.8 de	238.46
	1.0	8.6 def	230.77
1.0	0.25	10.0 cd	284.62
	0.5	11.0 bc	323.08
	0.75	13.4 a	415.38
	1.0	7.2 fg	176.92
1.5	0.25	11.2 bc	330.77
	0.5	12.2 ab	369.23
	0.75	8.8 de	238.46
	1.0	8.0 efg	207.69
2.0	0.25	8.2 efg	215.38
	0.5	5.6 h	115.38
	0.75	7.0 g	169.23
	1.0	4.4 h	69.23
CV%		12	-
LSD value		1.291	-

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

4.1.2.5 Average length of shoot (cm)

With different concentrations of BAP + KIN, significant difference was found on the length of shoot (cm). The results have been presented in Figure 6. The highest (4.42 cm) length of shoot was noticed from the 1.5 mg/L BAP + 0.25 mg/L

KIN which was statistically similar with 1.0 mg/L BAP + 1.0 mg/L KIN (4.40) and 1.0 mg/L BAP + 0.75 mg/L KIN (4.38), whereas the lowest was 3.30 cm in control.

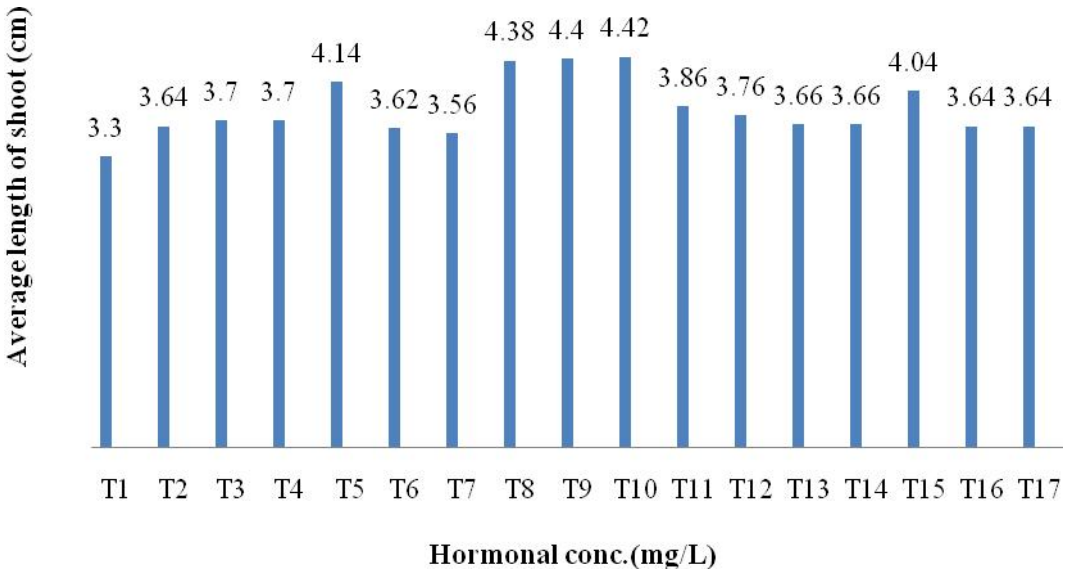


Figure 6: Effect of various combinations of BAP along with KIN on length of shoot (cm) in *Aloe vera* L. at 9 WAI

Hormonal conc. (mg/L):

T_1 = Nil			
T_2 = 0.5+0.25	T_6 = 1.0+0.25	T_{10} = 1.5+0.25	T_{14} = 2.0+0.25
T_3 = 0.5+0.5	T_7 = 1.0+0.5	T_{11} = 1.5+0.5	T_{15} = 2.0+0.5
T_4 = 0.5+0.75	T_8 = 1.0+0.75	T_{12} = 1.5+0.75	T_{16} = 2.0+0.75
T_5= 0.5+1.0	T_9 = 1.0+1.0	T_{13} = 1.5+1.0	T_{17} = 2.0+1.0

4.1.3 The combine effect of BAP + IBA on multiple shoot proliferation

The results of the combined effect of different concentrations of BAP + IBA have been presented under following headings with Tables 4-6, Figure 7 and Plate 6.

4.1.3.1 Percent of explant showing shoot induction

There was significant influence of BAP + IBA concentrations on percent of explant showing shoot induction. The highest percentage (85%) of shoot induction was found in treatment 1.0 mg/L BAP + 0.5 mg/L IBA and the lowest percentage (35%) was induced in hormone free media (Table 4).

Table 4. Combined effect of BAP and IBA on percent of explant showing shoot induction

BAP (mg/L)	IBA (mg/L)	Number of explant inoculated	Number of explant initiated shoot	Percent of explant showing shoot induction
MS-Control		20	7	35
0.5	0.25	20	9	45
	0.5	20	10	50
	0.75	20	13	65
	1.0	20	14	70
1.0	0.25	20	14	70
	0.5	20	17	85
	0.75	20	15	75
	1.0	20	16	80
1.5	0.25	20	15	75
	0.5	20	15	75
	0.75	20	14	70
	1.0	20	11	55
2.0	0.25	20	10	50
	0.5	20	12	60
	0.75	20	11	55
	1.0	20	13	65

4.1.3.2 Days to shoot induction

Variations were observed among different concentrations of BAP + IBA on days to shoot induction. The maximum days to shoot induction was recorded in control (41 days) and 1.0 mg/L BAP + 0.5 mg/L IBA required minimum 13.6 days (Table 5).

Table 5. Combined effect of BAP and IBA on shoot induction potentiality

BAP	IBA	Shoot induction potentiality

(mg/L)	(mg/L)	Days for shoot induction	Number of shoots per explant			% increase over control (9 WAI)
			3 WAI	6 WAI	9 WAI	
MS-Control		41.0 i	1.0 f	1.2 i	1.4 k	-
0.5	0.25	18.8 cd	1.0 f	2.6 fgh	4.6 j	228.57
	0.5	14.8 ghij	1.2 ef	3.2 efg	6.6 fgh	371.42
	0.75	15.6 fghi	1.6 de	3.6 cde	8.4 de	500.00
	1.0	14.2 hij	1.4 ef	3.2 efg	7.6 ef	442.86
1.0	0.25	17.4 def	1.6 de	4.4 bc	12.4 b	785.71
	0.5	13.6 j	3.2 a	6.6 a	14.8 a	957.14
	0.75	16.0 efgh	2.6 b	4.6 b	11.0 c	685.71
	1.0	15.6 ghi	2.4 bc	3.4 def	7.6 ef	442.86
1.5	0.25	14.8 ghij	2.2 bc	4.2 bcd	9.4 d	571.43
	0.5	20.6 b	2.0 cd	2.8 efgh	6.8 fg	384.71
	0.75	20.0 bc	1.6 de	3.6 cde	9.2 d	557.14
	1.0	18.8 cd	1.0 f	2.4 gh	7.4 ef	428.57
2.0	0.25	17.6 de	1.0 f	2.8 efgh	6.2 fghi	342.85
	0.5	13.8 ij	1.0 f	2.0 hi	4.8 ij	242.86
	0.75	16.2 efg	1.0 f	2.4 gh	5.2 hij	271.43
	1.0	16.0 efgh	1.0 f	2.2 h	5.6 ghij	300.00
CV%		7.05	23.83	20.59	14.19	-
LSD value		1.596	0.4739	0.8438	1.359	-

*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.

4.1.3.3 Number of shoots per explant

There was significant influence of different concentrations of BAP + IBA on the number of shoots per explant. Data were recorded after 3, 6 and 9 weeks of culture on MS media. The results have been presented in Table 5 and Plate 6. 1.0 mg/L BAP + 0.5 mg/L IBA gave the highest number of shoots (3.2, 6.6 and 14.8 at 3 WAI, 6WAI and 9WAI) respectively whereas the lowest number of shoots (1.0, 1.2 and 1.4 at 3WAI, 6WAI and 9WAI respectively) was found with hormone free media. Aggarwal and Barna (2004), observed 4.08 shoots per explant in media containing BA 1.0mg/L and IBA 0.2 mg/L on 4 weeks of culture. Chukwujekwu (2001) reported 11 shoots per explant from the combinations of 1.0mg/l BA + 1.0 mg/l IBA. Bhandari *et al.* (2010) reported 3.3 shoots per explant in combination with BAP 1.0 mg/L + IBA 0.2 mg/L after four weeks of culture.

6A **6B**

6C

Plate 6: The highest number of shoots on MS media supplemented with of 1.0 mg/L BAP + 0.5 mg/L IBA of *Aloe vera* L. (A) after 3 weeks, (B) after 6 weeks and (C) after 9 weeks of inoculation

4.1.3.4 Number of leaves per explant

The number of leaves per explant was significantly different according to the various concentration of BAP + IBA supplemented. The results have been presented in Table 6. The maximum number of leaves per explant (24.4) was noticed from 1.0 mg/L BAP + 0.5 mg/L IBA which was statistically similar with 1.0 mg/L BAP + 0.75mg/L IBA (23.2) and 1.5 mg/L BAP + 0.25 mg/L IBA (22.6) and different from all other treatment, whereas the minimum 2.6 leaves in control.

Table 6. Combined effect of BAP and IBA on the number of leaves per explant in *Aloe vera* L. at 9 WAI

BAP (mg/L)	IBA (mg/L)	No. of leaves per explant	% increase over control
MS-Control		2.6 h	-
0.5	0.25	9.0 g	246.15
	0.5	14.0 d	438.46
	0.75	17.2 c	561.54
	1.0	14.2 d	446.15
1.0	0.25	21.4 b	723.08
	0.5	24.4 a	838.46
	0.75	23.2 ab	792.31
	1.0	14.6 d	461.54
1.5	0.25	22.6 ab	769.23
	0.5	13.4 de	415.39
	0.75	17.2 c	561.54
	1.0	14.2 d	446.15
2.0	0.25	11.6 ef	346.15
	0.5	9.00 g	246.15
	0.75	9.80 fg	276.92
	1.0	10.4 fg	300.00
CV%		10.56	-
LSD value		1.950	-

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.

4.1.3.5 Average length of shoot (cm)

With different concentration of BAP + IBA, significant influence was found on the average length of shoot (cm). The results have been presented in Figure 7. The highest average length of shoot (6.76 cm) was noticed from the 1.5 mg/L BAP + 0.25 mg/L IBA which was statistically similar with 1.0 mg/L BAP + 0.5 mg/L IBA (6.72 cm) and 1.0 mg/L BAP + 0.25 mg/L IBA (6.44 cm) and different from all other treatment, whereas the lowest was 3.30 cm in control.

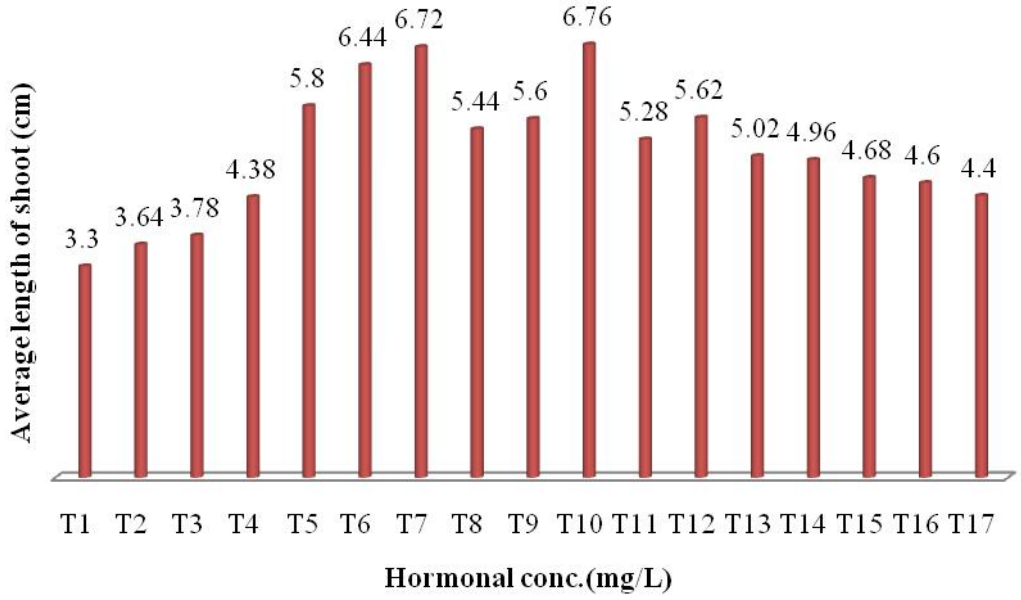


Figure 7: Effect of various combinations of BAP along with IBA on the length of shoot (cm) of *Aloe vera* L. at 9 WAI

Hormonal conc. (mg/L):

T_1 = Nil			
T_2 = 0.5+0.25	T_6 = 1.0+0.25	T_{10} = 1.5+0.25	T_{14} = 2.0+0.25
T_3 = 0.5+0.5	T_7 = 1.0+0.5	T_{11} = 1.5+0.5	T_{15} = 2.0+0.5
T_4 = 0.5+0.75	T_8 = 1.0+0.75	T_{12} = 1.5+0.75	T_{16} = 2.0+0.75
T_5= 0.5+1.0	T_9 = 1.0+1.0	T_{13} = 1.5+1.0	T_{17} = 2.0+1.0

4.1.4 The combine effect of BAP + NAA on multiple shoot proliferation

The result of the combined effect of different concentrations of BAP + NAA has been presented under following headings with Tables 7-8, figures 8-9 and Plate 7.

4.1.4.1 Percent of explant showing shoot induction

There was significant variation of BAP + NAA combination on percent of explant showing shoot induction. Maximum percentage (80%) of shoot induction was induced in treatment 1.5 mg/L BAP + 1.0 mg/L NAA and 1.5 mg/L BAP + 0.5 mg/L NAA. Minimum percentage (35%) was induced in hormone free media (Figure 8). Baksha *et al.* (2005) noticed 75 % shoot induction on MS supplemented with 2 mg/l BAP + 0.5 mg/l NAA.

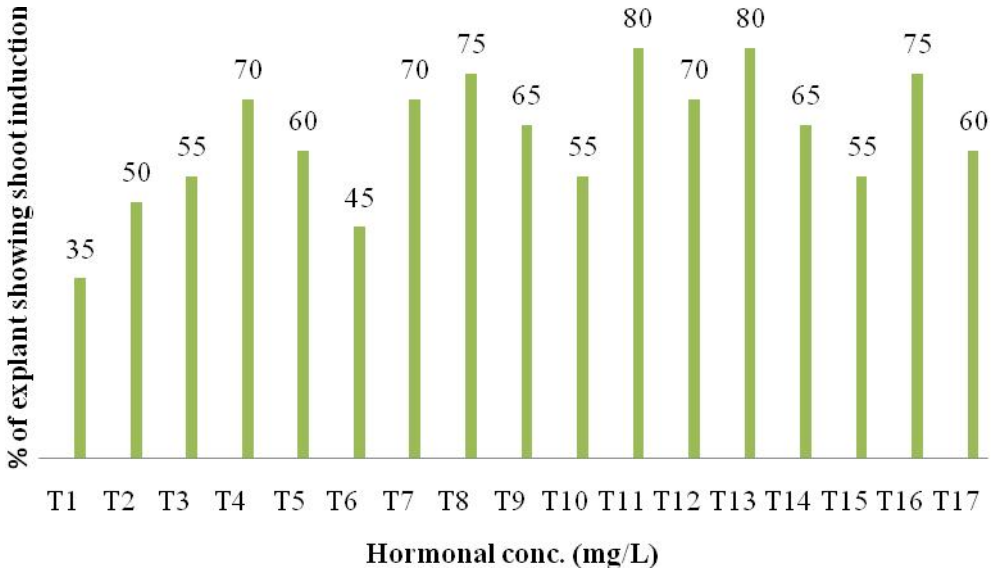


Figure 8: The combine effect of BAP + NAA on percent of explant showing shoot induction

Hormonal conc. (mg/L):

T_1 = Nil			
T_2 = 0.5+0.25	T_6 = 1.0+0.25	T_{10} = 1.5+0.25	T_{14} = 2.0+0.25
T_3 = 0.5+0.5	T_7 = 1.0+0.5	T_{11} = 1.5+0.5	T_{15} = 2.0+0.5
T_4 = 0.5+0.75	T_8 = 1.0+0.75	T_{12} = 1.5+0.75	T_{16} = 2.0+0.75
T_5= 0.5+1.0	T_9 = 1.0+1.0	T_{13} = 1.5+1.0	T_{17} = 2.0+1.0

4.1.4.2 Days to shoot induction

Variations were observed among different concentration of BAP + NAA on days to shoot induction. The maximum days to shoot induction was recorded in control (41 days) and 1.5 mg/L BAP + 1.0 mg/L NAA required minimum 14.4 days (Table 7). Baksha *et al.* (2005) noticed the lateral buds developed into shoot 10 - 15 days after inoculation on MS supplemented with 2 mg/L BAP + 0.5 mg/L NAA.

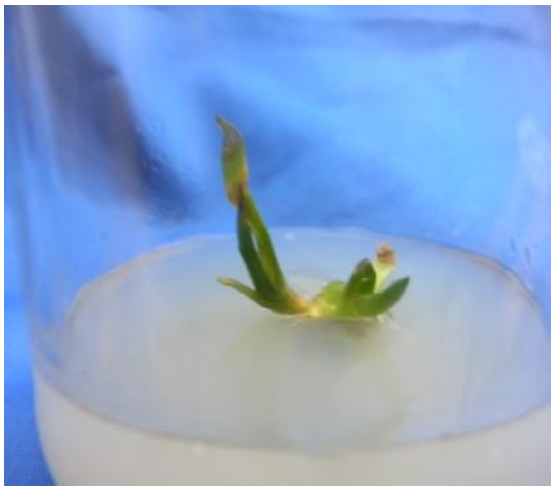
Table 7. Combined effect of BAP and NAA on shoot induction potentiality

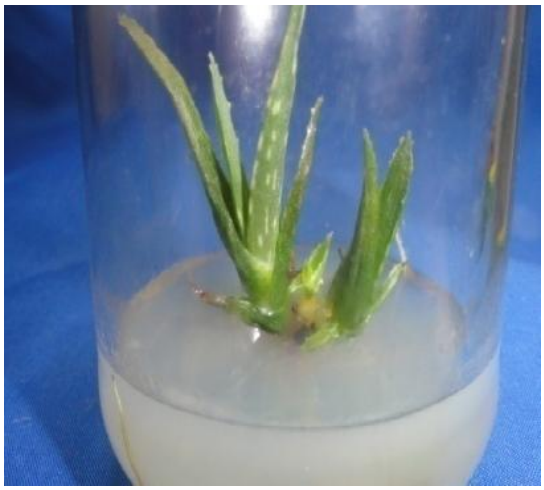
BAP (mg/L)	NAA (mg/L)	Shoot induction potentiality				
		Days for shoot induction	Number of shoots per explant			% increase over control (9 WAI)
			(3 WAI)	(6 WAI)	(9 WAI)	
MS-Control		41.0 i	1.0 d	1.2 g	1.4 h	-
0.5	0.25	24.6 b	1.0 d	1.4 fg	3.4 g	142.85
	0.5	24.0 b	1.0 d	1.6 efg	3.6 fg	157.14
	0.75	21.6 cd	1.0 d	2.6 cd	4.6 ef	228.57
	1.0	22.0 c	1.0 d	2.0 defg	4.0 efg	185.71
1.0	0.25	20.2 e	1.0 d	3.4 bc	4.8 de	242.86
	0.5	20.6 de	1.4 cd	3.8 b	6.4 bc	357.14
	0.75	17.6 f	1.0 d	3.6 b	6.6 bc	371.43
	1.0	17.2 f	1.0 d	2.4 de	7.4 b	428.57
1.5	0.25	15.6 g	1.2 cd	2.6 cd	7.0 b	400.00
	0.5	15.6 g	1.6 c	3.8 b	7.2 b	414.28
	0.75	15.4 gh	2.4 ab	4.0 b	7.4 b	428.57
	1.0	14.4 h	2.8 a	5.0 a	8.6 a	514.28
2.0	0.25	18.0 f	2.2 b	3.4 bc	4.2 efg	200.00
	0.5	20.6 de	1.4 cd	2.2 def	5.0 de	257.14
	0.75	17.4 f	1.4 cd	1.8 defg	5.8 cd	314.29
	1.0	15.6 g	1.2 cd	1.6 efg	6.8 bc	385.71
CV%		4.22	26.50	23.84	14.19	-
LSD value		1.069	0.4637	0.8218	1.359	-

*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.

4.1.4.3 Number of shoots per explant

There was significant influence of different concentrations of BAP + NAA on the number of shoots per explant. Data were recorded after 3, 6 and 9 weeks of culture on MS media. The results have been presented in Table 7 and Plate 7. A combination of 1.5 mg/L BAP + 1.0 mg/L NAA gave the highest number of shoots (2.8, 5.0 and 8.6 at 3 WAI, 6 WAI and 9 WAI respectively) which was statistically different from all other hormonal concentrations whereas the lowest number of shoots (1.0 1.2 and 1.4 at 3 WAI, 6 WAI and 9 WAI respectively) was found with hormone free media (Table 7). Biswas *et al.* (2013) achieved the best proliferation of average number of shoots per explant was 7.8 for the medium containing of 2 mg/L BA with 0.5 mg/L NAA after 8 weeks. Chukwujekwu (2001) reported 9 shoots per explant BA 1.5 mg/L along with 0.1 mg/L NAA.





7A **7B**

7C

Plate 7: Shoot proliferation of *Aloe vera* on MS media supplemented with 1.5 mg/L BAP + 1.0 mg/L NAA. (A) after 3 weeks, (B) after 6 weeks and (C) after 9 weeks of inoculation

4.1.4.4 Number of leaves per explant

With different concentrations of BAP + NAA, significant influence was found on the number of leaves. The highest 16.8 leaves per explant were recorded with 1.5 mg/L BAP + 1.0 mg/L NAA and the second highest was 15.2 leaves per explant in the 1.5 mg/L BAP + 0.75 mg/L NAA whereas minimum 2.6 leaves in case of lack of hormone (Figure 9).

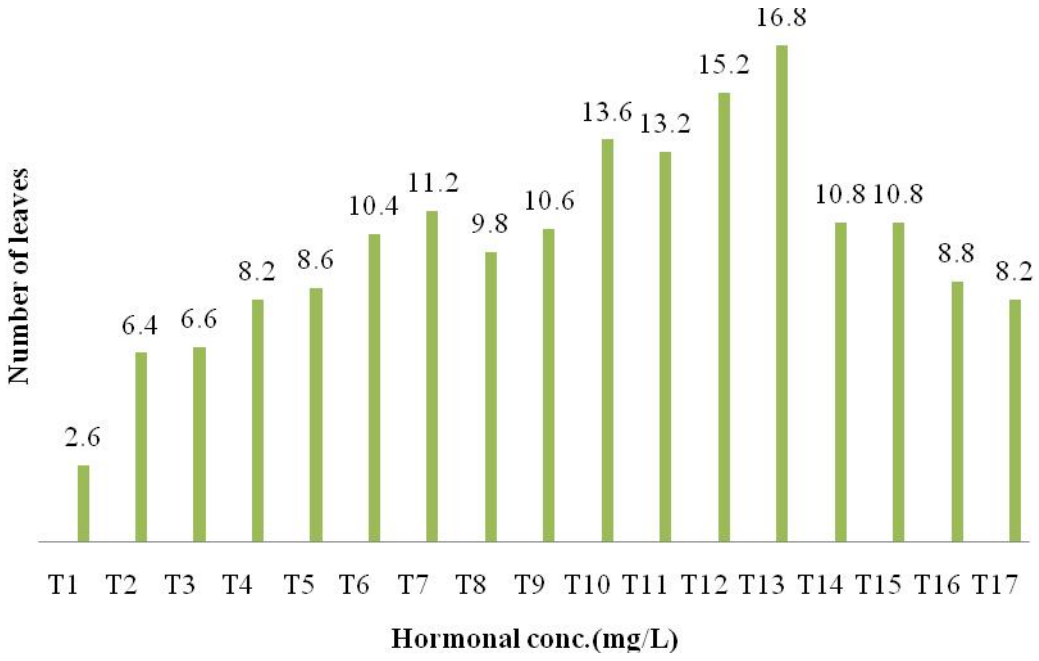


Figure 9: The combine effect of BAP + NAA on Number of leaves per explant at 9 WAI

Hormonal conc. (mg/L):

T_1 = Nil			
T_2 = 0.5+0.25	T_6 = 1.0+0.25	T_{10} = 1.5+0.25	T_{14} = 2.0+0.25
T_3 = 0.5+0.5	T_7 = 1.0+0.5	T_{11} = 1.5+0.5	T_{15} = 2.0+0.5
T_4 = 0.5+0.75	T_8 = 1.0+0.75	T_{12} = 1.5+0.75	T_{16} = 2.0+0.75
T_5= 0.5+1.0	T_9 = 1.0+1.0	T_{13} = 1.5+1.0	T_{17} = 2.0+1.0

4.1.4.5 Average length of shoot (cm)

The rate of shoot elongation was significantly different due to the influence of various concentrations of BAP + NAA supplemented. The results have been presented in Table 8. The highest length of shoot (9.62 cm) was noticed from the 1.5 mg/L BAP + 1.0 mg/L NAA and was statistically different from all other treatment, whereas the minimum 3.30 cm in control. Hashembadi and Kaviani (2010) obtained the best proliferation of shoot per explant (9.67) on medium supplemented with 0.5 mg/L BA + 0.5 mg/L NAA. Baksha *et al.* (2005) noticed the highest length of shoot (4.0 cm) on MS supplemented with 2 mg/L BAP + 0.5 mg/L NAA.

Table 8. Combined effect of BAP and NAA on the average length of shoot in *Aloe vera* L. at 9 WAI

BAP (mg/L)	NAA (mg/L)	Average length of shoot	% increase over control
MS-Control		3.30 i	-
0.5	0.25	5.92 j	79.39
	0.5	6.12 j	85.45
	0.75	6.06 j	83.64
	1.0	6.88 i	108.48
1.0	0.25	8.14 de	146.67
	0.5	7.10 hi	115.15
	0.75	6.92 hi	109.69
	1.0	8.72 c	164.24
1.5	0.25	7.76 ef	135.15
	0.5	8.34 cd	152.72
	0.75	9.14 b	176.97
	1.0	9.62 a	191.52
2.0	0.25	7.32 gh	121.82
	0.5	7.62 fg	130.90
	0.75	6.70 i	103.03
	1.0	6.80 i	106.06
CV%		8.52	-
LSD value		1.086	-

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.

4.1.5 The comparative performance of different growth hormone on shoot proliferation

The comparative performance of various growth hormone revealed variation among the different type of hormone used in present experiment and find out the best hormone dose for each of the parameter under consideration. The result has been presented in Tables 9-11, Figure 10 and Plate 8.

4.1.5.1 Percent of explant showing shoot induction

Significance variation was observed among the different type of hormone on percent of explant showing shoot induction. The maximum (85%) shoot induction was achieved with the 1.0 mg/L BAP + 0.5 mg/L IBA and minimum (60%) in case of 0.5 mg/L BAP (Figure 10).

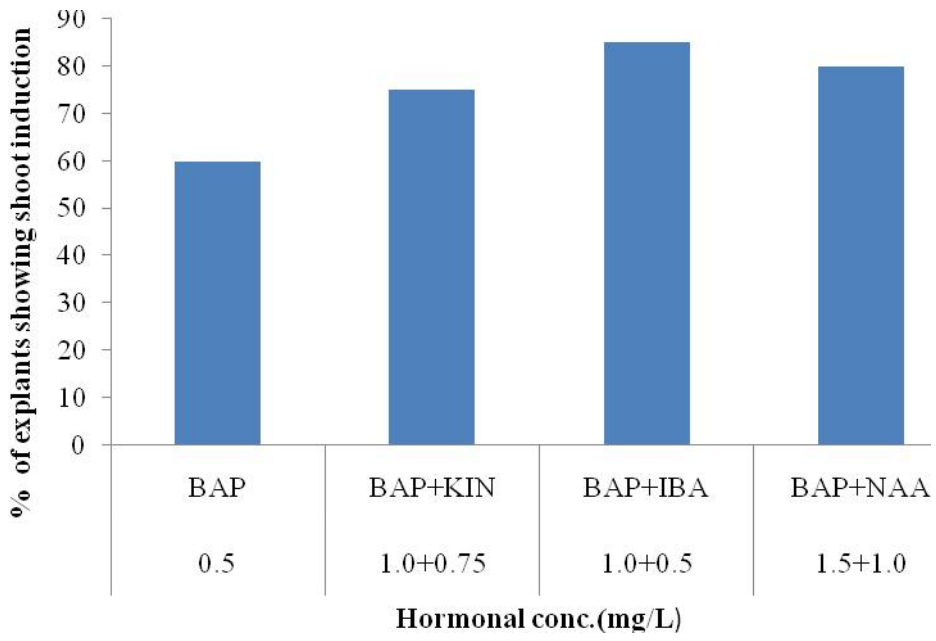


Figure 10: The comparative performance of growth hormone on percent of explant showing shoot induction

Figure revealed that a combined application of cytokinin and auxin had higher percent of explant showing shoot induction compared to a cytokinin (BAP) alone.

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Nayanakantha (2010) reported that BAP alone was less favorable for shoot induction after 4 weeks of culture. Similar results were also reported by other workers that combinations of cytokinins and auxins were better than cytokinins alone in the basal medium with respect to shoot proliferation (Hirimburegam and Gamage, 1995; Feng, 2000; Rout *et al.*, 2001; Velcheva *et al.*, 2005).

4.1.5.2 Days to shoot induction

The results of major effect of different types and combinations of hormone on Shoot induction potentiality have been presented Table 9. Significance variations were observed among these hormones. The highest number of 23 days to shoot induction was recorded in 0.5 mg/L BAP whereas the lowest number of 13.6 days required in media containing 1.0 mg/L BAP + 0.5 mg/L IBA.

Table 9. The comparative performance of growth hormone on shoot induction potentiality

Name of the Phyto-hormones	Phyto-hormones concentration (mg/L)	Shoot induction potentiality				
		Days for shoot induction	Number of shoots per explant			% increase over control (9 WAI)
			3 WAI	6 WAI	9 WAI	
BAP	0.5 + 0.0	23.0	1.0	2.4	4.2	200.00
BAP + KIN	1.0 + 0.75	18.8	1.0	3.8	7.4	428.57
BAP + IBA	1.0 + 0.5	13.6	3.2	6.6	14.8	957.14
BAP + NAA	1.5 + 1.0	14.4	2.8	5.0	8.6	514.28

4.1.5.3 Number of shoots per explant

In this study all the cytokinins singly or in combination with IBA or NAA showed multiple shoot production. The results of influence of different growth hormone on number of shoots per explant has been presented in Table 9. Significant influence was noticed at 3 WAI, 6 WAI and 9 WAI of data recording among these major hormonal doses. The highest number of shoots (3.2, 6.6 and 14.8 at 3 WAI, 6 WAI and 9 WAI respectively) was recorded in 1.0 mg/L BAP + 0.5 mg/L IBA and the lowest number of shoots (1.0, 2.4 and 4.2 at 3 WAI, 6 WAI and 9 WAI respectively) in 0.5 mg/L BAP. As previous workers reported that cytokinins alone or in combination with auxins positively influenced shoot proliferation in *Aloe* species (Hirimburegam and Gamage, 1995; Richwine *et al.,* 1995, Feng *et al.,* 2000). Baksha *et al.* (2005) also noticed comparatively a lower number of adventitious shoot were observed in the medium containing BAP alone.

4.1.5.4 Number of leaves per explant

The results of comparative study of major hormonal doses on number of leaves per explant have been presented in Table 10. Cytokinin level produced a significant response upon the number of shoot per explant and also showed influence on production of the numbers leaves per explant. The highest number of leaves (24.4) were found with 1.0 mg/L BAP + 0.5 mg/L IBA and the lowest (7.6) in 0.5 mg/L BAP.

Table 10. The comparative performance of growth hormone on number of leaves per explant

Name of the Phytohormones	Phytohormones concentration (mg/L)	No. of leaves per explant	% increase over control
BAP	0.5 + 0.0	7.6	192.31
BAP + KIN	1.0 + 0.75	13.4	415.38
BAP + IBA	1.0 + 0.5	24.4	838.46
BAP + NAA	1.5 + 1.0	16.8	546.15

4.1.5.5 Average length of shoot (cm)

There was significance influence of different hormone combinations and concentrations on average Length of shoot (cm). The comparative results of different

growth hormones have been presented in Table 11 and Plate 8. The maximum (9.62 cm) average length of shoot was obtained with BAP 1.5 mg/L+1.0 mg/L NAA whereas the minimum (4.42 cm) average length of shoot in 1.5mg/L BAP+0.25 mg/L KIN.

Table 11. The comparative performance of growth hormone on average length of shoot (cm)

Name of the Phytohormones	Phytohormones concentration (mg/L)	Average length of shoot (cm)	% increase over control
BAP	1.0 + 0.0	4.46	35.15
BAP + KIN	1.5 + 0.25	4.42	33.93
BAP + IBA	1.5 + 0.25	6.76	104.45
BAP + NAA	1.5 + 1.0	9.62	191.52

The results showed that combinations of BAP + NAA and BAP+IBA were better than BAP alone or in combination with KIN in the basal MS medium in terms of increasing the shoot elongation. This could be attributed to the balancing of the cytokinin/auxin ratio (Chukwujekwu, 2001).

8A

8B

Plate 8: The longest shoot of *Aloe vera* produced on MS media supplemented with 1.5 mg/L BAP + 1.0 mg/L NAA at 9 WAI

4.2 Experiment 2. Root formation in *Aloe vera* L.

To develop root, the regenerated shoot excised and transferred to rooting media supplemented with IBA and NAA separately. The results of experiment have been presented under different heading utilizing Figures 11-15, Tables 12-14 and Plates 9-12.

4.2.1 Percent of explant showing root induction

There were considerable variation among growth regulators on percent of explant showing root induction. The results have been shown in Figure 11. The highest percentage (95%) of root induction was recorded with 3.0 mg/L IBA, whereas 90% root induction was found in 2.0 mg/L NAA. The lowest percentage (30%) of root induction was recorded in media lack of plant growth regulator.

Dwivedi *et al.* (2014) found the highest percentage of root induction (80%) in MS medium supplemented with IBA (0.5 mg/ L). Similar result was reported by Abrie and Staden (2001) in *Aloe vera*. Zakia *et al.* (2013) reported best rooting (84.67%) was found in medium supplemented with 1.5 mg/L of indole butyric acid (IBA).While

Baksha *et al.* (2005) noticed 75% root induction in IBA 1.5 mg/L and about 95% rooting from micro-shoot cultured on half strength MS supplemented with NAA (0.5 mg/L). Biswas *et al.* (2013) observed 90 % root induction in NAA 0.5 mg/L.

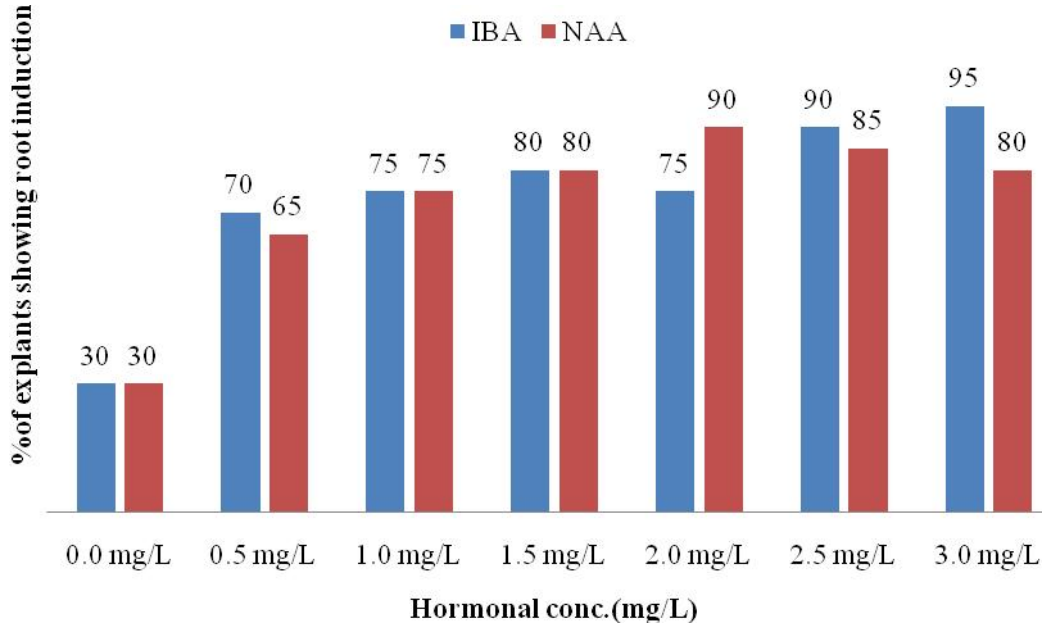


Figure 11: Effects of IBA and NAA on percent of explant showing root induction

However, the higher concentration above 2.0 mg/L exerted its inhibitory effect on the rooting percentage in media with NAA (Figure 11). The inhibiting effect might be because higher concentration induces the higher level of degradative metabolites in tissue, thus, blocking the regeneration process (Baker and Wetstein, 2004). Numerous studies also supported the usefulness of IBA as the most effective auxin in various plants rhizogenesis as compare to NAA (Benelli *et al.*, 2001; Tanimoto, 2005). They also found IBA is the preferred auxin for the induction of root formation because it is much more potent than IAA or synthetic auxins.

4.2.2 Days to root induction

Hormonal concentration has significant level of variation on days for root induction. The maximum 25 days to root induction was required in media lack of growth regulator. Minimum 10.6 days in case of 2.5 mg/L NAA and 12.6 days was required by 3.0 mg/L IBA (Figure 12). Baksha *et al.* (2005) noticed root began to emerge from the tenth day of in the medium with 0.5 mg/l of NAA.

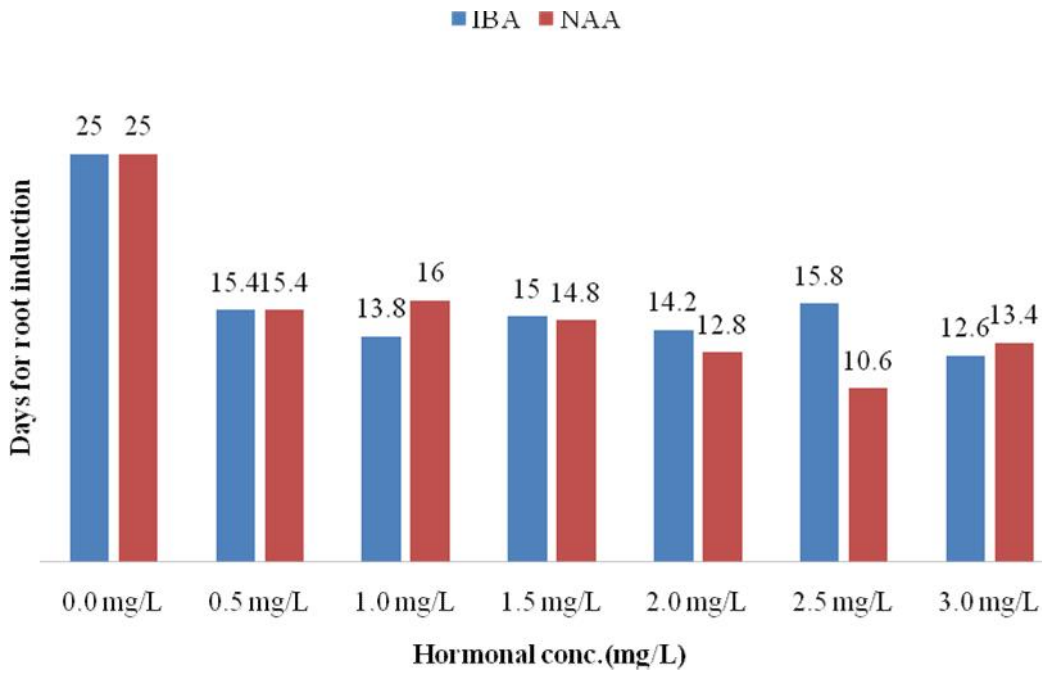


Figure 12: Effects of IBA and NAA on days to root induction

4.2.3 Number of roots per explant

To evaluate the response and effectiveness of IBA and NAA on the number of roots, a range of treatment (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0) was applied and significant variations were observed during data recording at 3 WAI, 4 WAI and 5 WAI. Root regenerated in the medium containing IBA were thin and long compared to root from NAA which were relatively thick and strong (Plate 9, 10) even during initial stage of development.

In case of IBA, the highest number of roots (3.6) per explant was recorded in 3.0 mg/L IBA at 3 WAI, which was not statistically different from 2.5 mg/L IBA (3.4). But the highest number of roots (7.4 and 12.4 at 4 WAI and 5WAI respectively) were found only with 3.0 mg/L IBA. The minimum number of roots (0.0, 1.8 and 2.4 at 3 WAI, 4 WAI and 5 WAI respectively) were obtained in control (Table 12 and Plate 9).

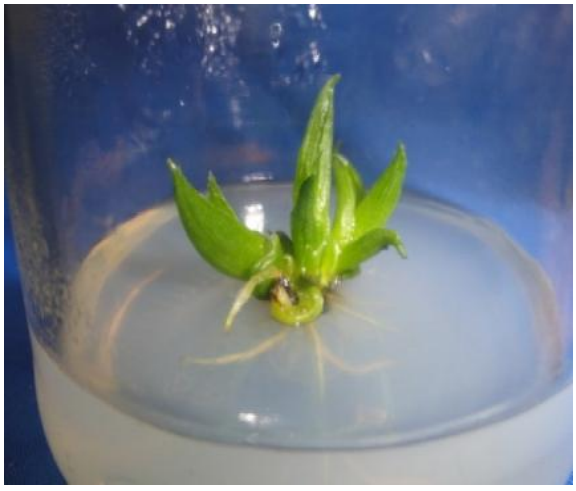
Dwivedi *et al.* (2014) found 10 roots in medium with IBA (0.5 mg/ L) in 8 weeks of time. Bhandari *et al.* (2010) reported 2.6 roots in IBA 0.2 mg/L after 15 days of culture. Baksha *et al.* (2005) noticed 3.2 roots per explant in IBA 1.5 mg/L.

Table 12. Effect of IBA on number of roots *in Aloe vera* L.

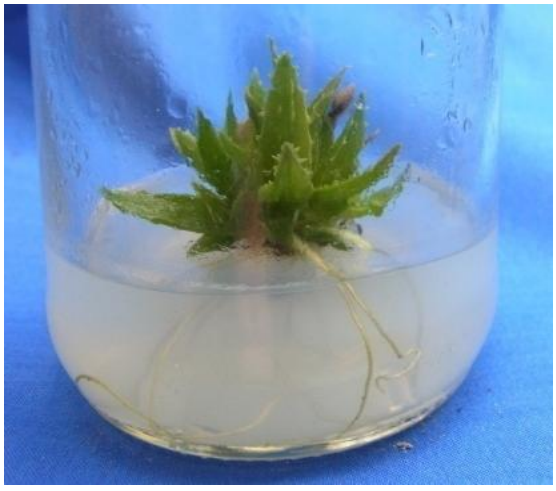
IBA (mg/L)	No. of roots per explant			% increase over control
	3 WAI	4 WAI	5 WAI	(5 WAI)
MS-Control	-	1.8 f	2.4 f	-
0.5	1.6 c	2.6 e	6.4 d	166.67
1.0	2.4 bc	4.4 c	5.2 e	116.66
1.5	3.2 ab	3.6 d	8.4 c	250.00
2.0	3.0 ab	5.4 b	5.6 de	133.33
2.5	3.4 a	4.6 c	10.4 b	333.33

3.0	3.6 a	7.4 a	12.4 a	416.67
CV%	26.20	12.56	9.02	-
LSD value	0.8336	0.6928	0.8485	-

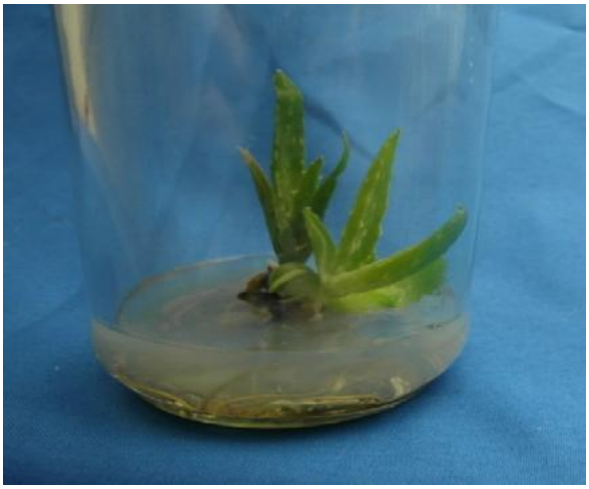
*WAI=Weeks After Inoculation. Values in the column are the means of five replicates. In a column, mean values with the same letters are not statistically different from each other at 5% probability by DMRT.



9A



9B



9C

Plate 9: The highest number of roots on MS media supplemented with 3.0 mg/L IBA of *Aloe vera* L. (A) after 3 weeks, (B) after 4 weeks and (C) after 5 weeks of inoculation

In case of NAA, the highest number of roots (3.4, 6.4 and 9.6 at 3 WAI, 4 WAI and 5 WAI respectively) were found with 2.0 mg/L IBA which was statistically different from other treatment. The lowest number of roots (0, 1.8 and 2.4 at 3 WAI, 4 WAI and 5 WAI respectively) were obtained in control (Figure 13 and Plate 10).

Biswas *et al.* (2013) achieved highest average number of roots per explant was 5.2 produced in 0.5 mg/L Napthaleneacetic acid concentration after 15 days of culture of microshoot on rooting medium. Daneshvar *et al.* (2013) noticed 6.8 roots with 0.5 mg/L NAA. Hashembadi and Kaviani (2010) obtained the largest number of roots (9.71) was obtained on medium supplemented with 0 mg/L IBA + 1 mg/L NAA. Baksha *et al.* (2005) noticed the highest number of roots per root was 4.8 with 0.5 mg/L NAA.

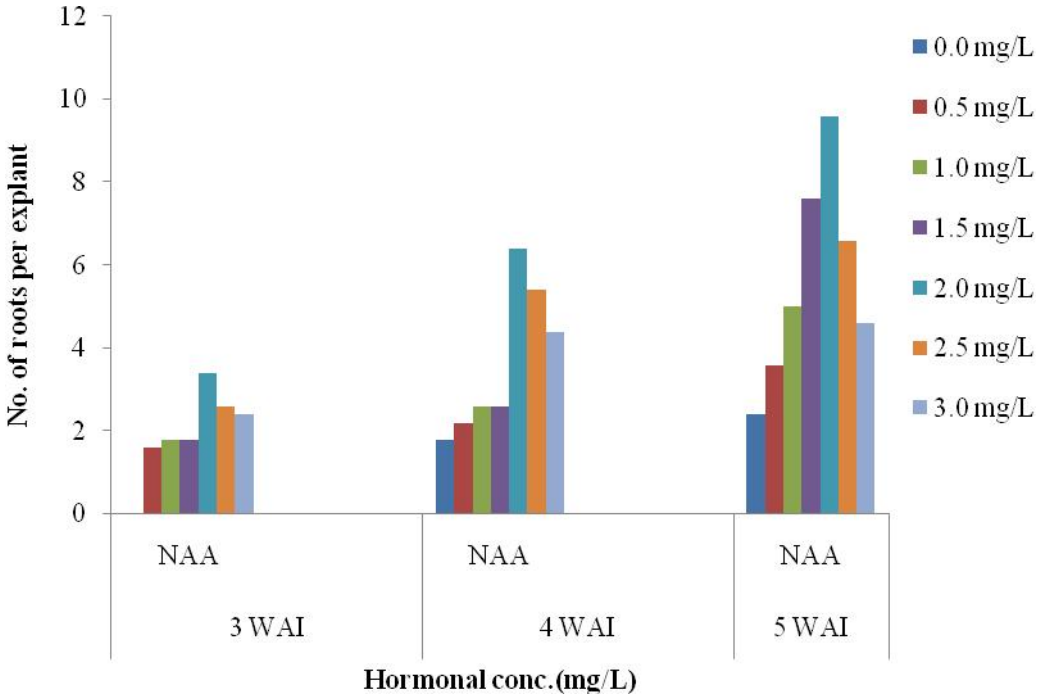
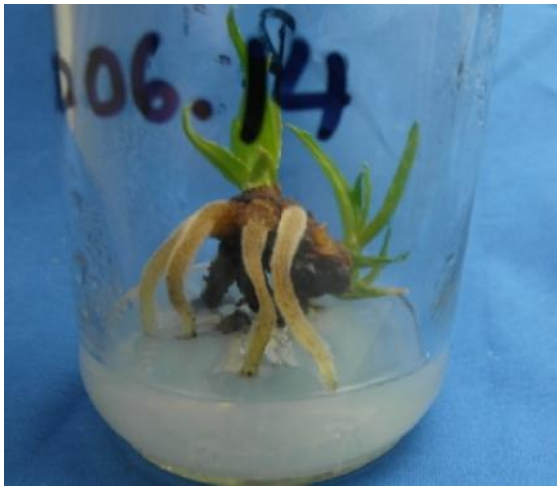


Figure 13: Effects of NAA on number of roots at 3 WAI, 4 WAI and 5 WAI *in Aloe vera* L.

10A **10B**



10C

Plate 10. Root formation of *Aloe vera* on MS media supplemented with 2.0 mg/L NAA. (A) after 3 weeks, (B) after 4 weeks and (C) after 5 weeks of inoculation

4.2.4 Average length of root (cm)

Average length of root per explant (cm) was greatly regulated by the different concentrations of both IBA and NAA. The maximum average root length (10.24 cm) was obtained from 2.0 mg/L IBA (Table 13 and Plate 11A) and 7.66 cm in case of 2.5 mg/L NAA (Figure 14 and Plate 11B). The minimum 1.86 cm average length of root (cm) was in control.

Jafari and Hamidoghli (2009) explained that concentration of 2 mg/L IBA has given a bigger number of root and the maximum root length. Dwivedi *et al.* (2014) found length 6 cm were obtained in medium with IBA 0.5 mg/ L in 8 weeks of time. Daneshvar *et al.* (2013) noticed 6.32 cm root length in 1.0 mg/L IBA. Hashembadi

and Kaviani (2010) obtained the highest number (8.75 cm) root were achieved on medium supplemented with 1 mg/L IBA + 1 mg/L NAA. Baksha *et al.* (2005) noticed the highest average length of 3.5 cm with 0.5 mg/L NAA and 2.2 cm in IBA 1.5 mg/L.

Table 13. Effect of IBA on average length of root (cm) *in Aloe vera* L. at 5 WAI

IBA (mg/L)	Average length of root (cm)	% increase over control
MS-Control	1.86 g	-
0.5	7.08 d	280.65
1.0	6.44 e	246.24
1.5	6.04 f	224.73
2.0	10.24 a	450.54
2.5	9.90 b	432.26
3.0	7.60 c	308.60
CV%	3.65	-
LSD value	0.3328	-

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.

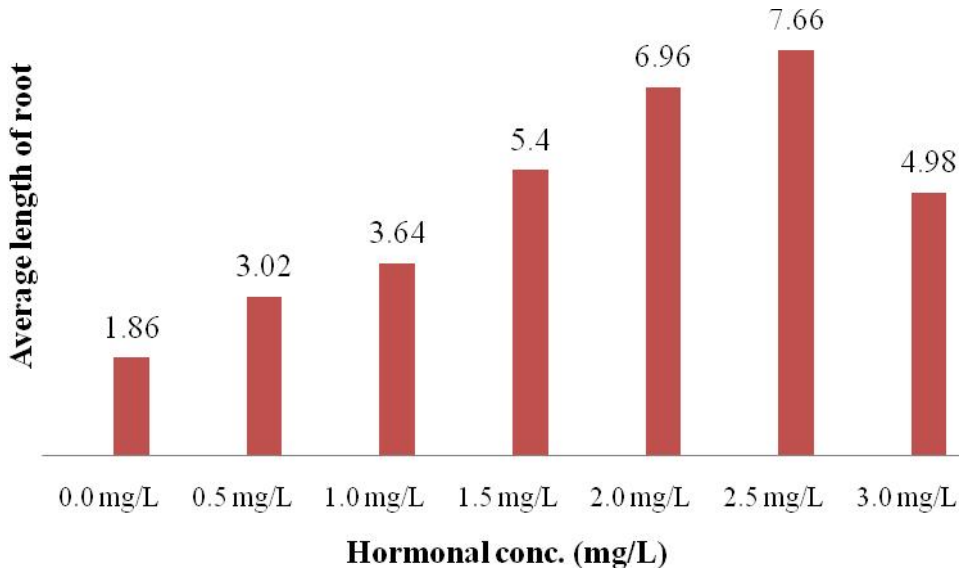
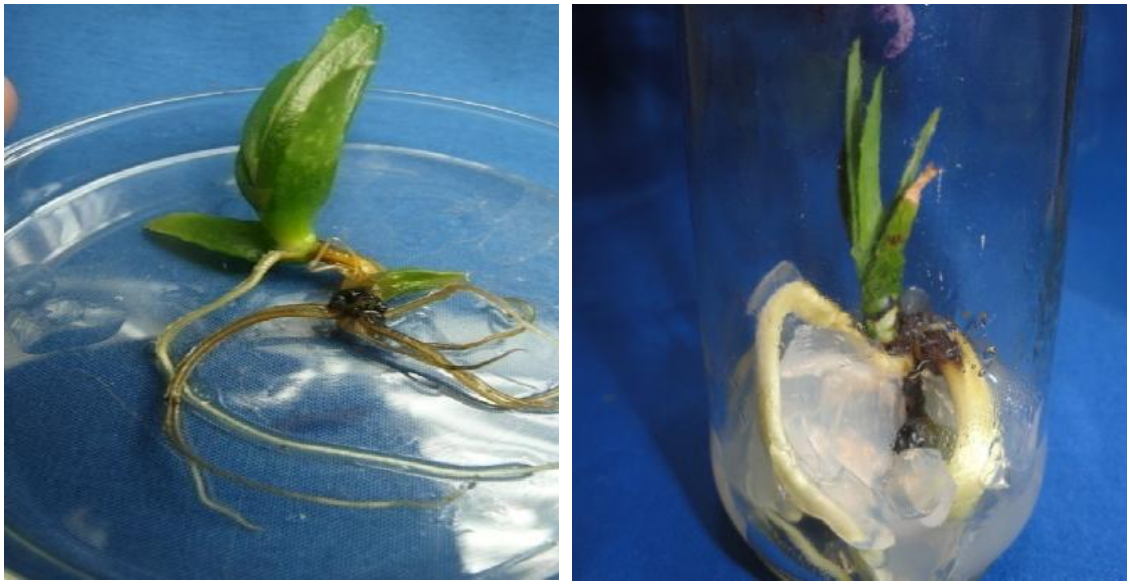


Figure 14: Effects of NAA on average length of root (cm) *in Aloe vera* L. at 5 WAI



11A **11B**

Plate 11. The longest root of *Aloe vera* obtained on MS media supplemented with (A) 2.0 mg/L IBA and (B) 2.5 mg/L NAA at 5 WAI

4.2.5 Diameter of root (mm)

Different treatments of IBA and NAA played a vital role on diameter of root (mm). They were statistically dissimilar. The thickest root was 5.374 mm in diameter with 2.5 mg/L NAA (Table 14 and Plate 12B), whereas root with 1.422 mm in diameter was observed in culture media containing 3.0 mg/L IBA (Figure 15 and Plate 12A). The least root diameter (0.576 mm) was reported from growth hormone free culture media. Hashemabadi and Kaviani (2010) found the maximum 4.3 mm diameter of root on medium supplemented with 1 mg/L IBA + 1 mg/L NAA.

Table 14. Effect of NAA on diameter of root (mm) *in Aloe vera* L. at 5 WAI

NAA (mg/L)	Diameter of root (mm)	% increase over control
MS-Control	0.576 g	-
0.5	2.786 f	383.69
1.0	3.364 e	484.03
1.5	3.682 d	539.24
2.0	4.308 c	647.92
2.5	5.374 a	832.97
3.0	4.898 b	750.35
CV%	6.11	-

LSD value	0.0819	-

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.

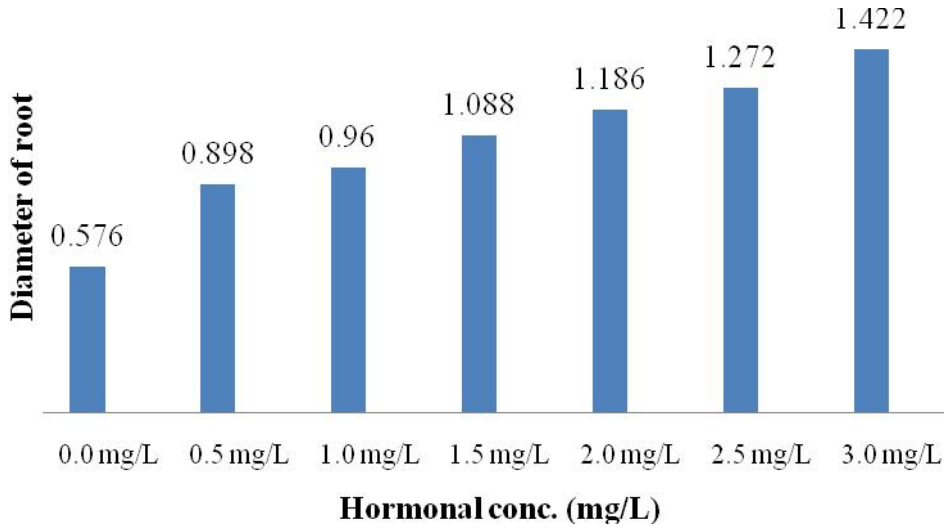


Figure 15: Effects of IBA on diameter of root (mm) *in Aloe vera* L. at 5 WAI

Root induced by a high concentration of NAA were thicker and the survival rate of such type of root was lower compared with IBA induced root (Plate 12). The NAA supplimented media caused an increasing of root diameter with the increase in hormonal concentration (Table 14).

12A **12B**

Plate 12. The thickest root of *Aloe vera* obtained on MS media supplemented with (A) 3.0 mg/L IBA and (B) 2.5 mg/L NAA at 5 WAI

4.3 Experiment 3. Acclimatization of plantlets

The results of acclimatization or "hardening-off' have been presented in Table 15 and Plate 13. After 35 days of culture on rooting media, the plantlets were taken for acclimatization.

Table 15. Survial rate of *in vitro* regenerated plants of *Aloe vera* L.

Acclimatization	No. of plants transplanted	Duration of observation	No. of plants survived	Survival rate (%)
In growth chamber	25	7days	25	100
In shade house	25	14 days	22	88
In field condition	22	30 days	20	90

The results of acclimatization showed that the 100% of plantlets were survived to growth chamber (Table 15 and Plate 13A). Then the plantlets were shifted to shade house with less humidity (70% RH) and indirect sunlight (Plate 13B). In the shade house, the top of the pots were covered with transparent plastic sheet and grew at room temperature for 14 days with periodic irrigation (2 days interval). In these conditions, the 88% of the plantlets survived (Table 15). After 3 weeks, the plantlets were transferred to the soil following depoting and poting into different pots of bigger size. The plants were watered periodically and upper layer of the soil mulched occasionally whenever necessary (Plate 13C). In open atmosphere, survival rate was 90% (Table 15). It was also revealed that regenerated plants were morphologically similar to the mother plant.

Aggarwal and Barna (2004) used soil and farmyard manure (1:1) for hardening, and plantlets transferred to the polyhouse, then 85% of the plantlets survived. After keeping them for initial 10 days in polyhouse, plants were transferred to shadehouse under less humidity. In shadehouse, 82% plantlets were survived. Dwivedi *et al.* (2014) found 83% the survival rate and the plants established well in 4-6 weeks of growth. Bhandari *et al.* (2010) observed the plantlets that were transferred to the plastic pots in poly house showed 90% survival and under shade house (50%) it was

found 80%. Baksha *et al.* (2005) reported that well-developed rooted plantlets were successfully transferred to the soil with 70% survival.

13A

13B

13C

Plate 13. Acclimatization of regenerated planted (A) in growth chamber, (B) in shade house and (C) in field condition

CHAPTER V

SUMMARY AND CONCLUTION

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 during the period from September, 2013 to July, 2014 to evaluate the effect of different plant growth regulators on *in vitro* multi shoot proliferation and root formation along with acclimatization for *in vivo* survival of *Aloe vera*.

Shoot tips of young lateral shoot (sucker) were used as explant for *in vitro* regeneration of *Aloe vera*. The major findings have been summarized below.

For percent of explant showing shoot induction was the highest (85%) at 1.0 mg/L BAP + 0.5 mg/L IBA and the lowest (60%) in case of 0.5 mg/L BAP.

Significance variation was observed among the hormone on days to shoot induction. The highest 23 days to shoot induction was recorded in 0.5 mg/L BAP whereas the lowest 13.6 days in media containing 1.0 mg/L BAP + 0.5 mg/L IBA.

The highest number of shoots (3.2, 6.6 and 14.8 at 3 WAI, 6 WAI and 9 WAI) respectively were recorded in 1.0 mg/L BAP + 0.5 mg/L IBA and the lowest

number of shoots (1.0, 2.4 and 4.2 at 3 WAI, 6WAI and 9WAI) respectively in only 0.5 mg/L BAP.

Cytokinin level produced a significant response upon the number of shoot per explant and also showed influence on production of the numbers leaves per explant. The highest number of leaves (24.4) was found with 1.0 mg/L BAP + 0.5 mg/L IBA and the lowest (7.6) in 0.5 mg/L BAP.

It revealed , the highest (9.62 cm) average length of shoot was observed with 1.5 mg/L BAP+1.0 mg/L NAA whereas the lowest (4.42 cm) average length of shoot in case of 1.5 mg/L BAP + 0.25 mg/L KIN.

The results showed, the highest percentage (95%) of root induction was recorded with 3.0 mg/L IBA. The lowest percentage (30%) of root induction was recorded in media lack of plant growth regulator.

The maximum 25 days to root induction was required in media devoid of growth regulator. The minimum 10.6 days was required in case of 2.5 mg/L NAA.

The highest number of roots (3.6) per explant was recorded in 3.0 mg/L IBA at 3 WAI, which was not statistically different with 2.5 mg/L IBA (3.4). But the highest number of roots (7.4 and 12.4 at 6 WAI and 9 WAI respectively) were found with 3.0 mg/L IBA. The lowest number of roots (0.0, 1.8 and 2.4 at 3 WAI, 6 WAI and 9 WAI) respectively were obtained in control.

The maximum average root length (10.24 cm) was obtained from 2 mg/L IBA. The minimum 1.86 cm average length of root per explant (cm) was in control. Different treatment of IBA and NAA play a vital role on diameter of root (mm). The thickest root was 5.374 mm in diameter with 2.5 mg/L NAA. The least root diameter (0.576 mm) was reported from growth hormone free culture media.

Regenerated plantlets showed, 100% survival during in growth chamber conditions and 88% in shade house stage of hardening and 90% in open atmosphere.

In conclusion, regenerated plants were found to be morphologically similar to the mother plant. *In vitro* regeneration protocol of *Aloe vera* has been developed. The results showed that combinations of BAP + IBA and BAP+NAA were better than all other hormones under study. Very thick roots induction was noticed in MS media supplemented with NAA. Regenerated plants were found to be morphologically similar to the mother plant. Present study showed that micropropagation is effective

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

CHAPTER VI
RECOMMANDATIONS

Following recommendations could be addressed based on the present experiment:

i. Further study can be done with different concentrations and combinations of auxins and cytokinines group of hormones for rapid proliferation of *Aloe vera.*

ii. In addition to shoot tip culture, meristem and root tip culture can be done.

iii. Callus induction can be done with 2.4-D or other callus induction hormone for large number of shoot induction.

iv. Influence of other factors (elicitors, antioxidants) such as ascorbic acid, activated charcoal and polyvinylpyrrolidone should be considered.

v. Biochemical test of regenerated *Aloe vera* may be done to extract the active ingredients (metabolites) of *Aloe vera.*

vi. To uncover the influence of genotype if any, research should be carried on with different type of genotype of *Aloe vare*.

CHAPTER VII
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CHAPTER VIII
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 BAP

	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability
Between	4	847.76	211.94	91.353	0.0
Within	20	46.40	2.32		
Total	24	894.16			
CV%	4.84%				
LSD value	2.009				

Appendix III. Analysis of variance on days to shoot induction with BAP+KIN

	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability
Between	16	1926	120.375	110.02	0.0
Within	68	74.4	1.094		

Total	84	2000.4			
CV%	4.25				
LSD value	1.320				

Appendix IV. Analysis of variance on days to shoot induction with BAP+IBA

	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability
Between	16	3180.776	198.799	124.249	0.0
Within	68	108.8	1.6		
Total	84	3289.576			
CV%	7.05				
LSD value	1.596				

Appendix V. Analysis of variance on days to shoot induction with BAP+NAA

	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability
Between	16	3101.624	193.851	270.121	0.0
Within	68	48.8	0.718		
Total	84				
CV%	4.22				
LSD value	1.069				

Appendix VI. Analysis of variance on number of shoots per explant at 3 WAI with BAP+IBA

	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability
Between	16	37.153	2.322	16.448	0.0
Within	68	9.6	0.141		
Total	84	46.753			
CV%	23.83				
LSD value	0.4739				

AppendixVII. Analysis of variance on number of shoots per explant at 3WAI with BAP+NAA

	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability
Between	16	24.988	1.562	11.543	0.0
Within	68	9.2	0.135		
Total	84	34.188			
CV%	26.5				
LSD value	26.50				
	0.4637				

Appendix VIII. Analysis of variance on number of shoots per explant at 6 WAI with BAP

	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability
Between	4	4.56	1.14	4.75	0.0074
Within	20	4.80	0.24		

Total	24	9.36			
CV%	26.62				
LSD value	0.6463				

Appendix IX. Analysis of variance on number of shoots per explant at 6 WAI with BAP+KIN

	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability
Between	16	65.647	2.103	9.426	0.0
Within	68	29.6	0.435		
Total	84	95.247			
CV%	26.33				
LSD value	0.8324				

Appendix X. Analysis of variance on number of shoots per explant at 6 WAI with BAP+IBA

	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability
Between	16	123.412	7.713	17.253	0.0
Within	68	30.400	0.447		
Total	84	153.812			
CV%	20.59				
LSD value	0.8438				

Appendix XI. Analysis of variance on number of shoots per explant at 6 WAI with BAP+NAA

	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability
Between	16	95.976	5.999	14.163	0.0
Within	68	28.8	0.424		
Total	84	124.776			
CV%	23.84				
LSD value	0.8218				

Appendix XII. Analysis of variance on number of shoots per explant at 9 WAI with BAP

	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability
Between	4	22.16	5.54	21.308	0.0
Within	20	5.2	0.26		
Total	24	27.36			
CV%	17.95				
LSD value	0.6727				

Appendix XIII. Analysis of variance on number of shoots per explant at 9 WAI with BAP+KIN

	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability
Between	16	219.647	13.728	18.821	0.0
Within	68	49.600	0.729		
Total	84	269.247			

CV%	18.95
LSD value	1.078

Appendix XIV. Analysis of variance on number of shoots per explant at 9 WAI with BAP+IBA

	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability
Between	16	807.788	50.487	43.567	0.0
Within	68	78.800	1.159		
Total	84	886.588			
CV%	14.19				
LSD value	1.359				

Appendix XV. Analysis of variance on number of shoots per explant at 9 WAI with BAP+NAA

	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability
Between	16	280.306	17.519	29.199	0.0
Within	68	40.800	0.600		
Total	84				
CV%	13.98				
LSD value	0.9776				

Appendix XVI. Analysis of variance on average length of shoot (cm) with BAP at 9 WAI

	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability
Between	4	4.626	1.157	21.905	0.0
Within	20	1.056	0.053		
Total	24	5.682			
CV%	5.82				
LSD value	0.3037				

Appendix XVII. Analysis of variance on average length of shoot (cm) with BAP+KIN at 9 WAI

	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability
Between	16	8.596	0.537	3.044	0.0007
Within	68	12.004	0.177		
Total	84	20.6			
CV%	10.97				
LSD value	0.5310				

Appendix XVIII. Analysis of variance on average length of shoot (cm) with BAP+IBA at 9 WAI

	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability
Between	16	84.521	5.283	27.898	0.0
Within	68	12.876	0.189		
Total	84	97.397			
CV%	8.56				
LSD value	0.5487				

Appendix XIX. Analysis of variance on average length of shoot (cm) with BAP+NAA at 9 WAI

	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability
Between	16	172.665	10.792	117.15	0.0
Within	68	6.264	0.092		
Total	84	178.929			
CV%	4.21				
LSD value	0.3828				

Appendix XX. Analysis of variance on number of leaves per explant with BAP at 9 WAI

	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability
Between	4	68.16	17.04	24.343	0.0
Within	20	14.00	0.700		
Total	24	82.16			
CV%	15.38				
LSD value	1.104				

Appendix XXI. Analysis of variance on number of leaves per explant with BAP+KIN at 9 WAI

	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability
Between	16	611.976	38.249	36.529	0.0
Within	68	71.200	1.047		
Total	84	683.176			
CV%	12				
LSD value	1.291				

Appendix XXII. Analysis of variance on number of leaves per explant with BAP+IBA at 9 WAI

	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability
Between	16	2761.294	172.581	72.263	0.0
Within	68	162.400	2.388		
Total	84	2923.694			
CV%	10.56				
LSD value	1.950				

Appendix XXIII. Analysis of variance on number of leaves per explant with BAP+NAA at 9 WAI

	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability
Between	16	943.647	58.978	79.573	0.0
Within	68	50.400	0.741		
Total	84	994.047			
CV%	8.52				
LSD value	1.086				

Appendix XXIV. Analysis of variance on days to root induction with IBA

	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability
Between	6	510.171	85.029	270.545	0.0
Within	28	8.800	0.314		
Total	34	518.971			
CV%	3.51				
LSD value	0.4742				

Appendix XXV. Analysis of variance on days to root induction with NAA

	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability
Between	6	633.371	105.562	263.905	0.0
Within	28	11.200	0.400		
Total	34	644.571			
CV%	4.10				
LSD value	0.8194				

Appendix XXVI. Analysis of variance on number of roots per explant at 3 WAI with IBA

	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability
Between	6	49.086	8.181	19.747	0.0
Within	28	11.6	0.414		
Total	34	60.686			
CV%	26.20				
LSD value	0.8336				

Appendix XXVII. Analysis of variance on number of roots per explant at 3 WAI with NAA

	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability
Between	6	33.486	5.581	24.417	0.0
Within	28	6.400	0.229		

Total	34				
CV%	24.61				
LSD value	0.6200				

Appendix XXVIII. Analysis of variance on number of roots per explant at 4 WAI with IBA

	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability
Between	6	102.686	17.114	59.9	0.0
Within	28	8	0.286		
Total	34	110.686			
CV%	12.56				
LSD value	0.6928				

Appendix XXIX. Analysis of variance on number of roots per explant at 4 WAI with NAA

	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability
Between	6	94.571	15.762	45.972	0.0
Within	28	9.6	0.343		
Total	34	104.171			
CV%	16.14				
LSD value	0.7587				

Appendix XXX. Analysis of variance on number of roots per explant at 5 WAI with IBA

	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability
Between	6	344.686	57.448	134.044	0.0
Within	28	12	0.429		
Total	34	356.686			
CV%	9.02				
LSD value	0.8485				

Appendix XXXI. Analysis of variance on number of roots per explant with NAA at 5 WAI

	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability
Between	6	182.971	30.495	92.812	0.0
Within	28	9.2	0.329		
Total	34	192.171			
CV%	10.18				
LSD value	0.7431				

Appendix XXXII. Analysis of variance on average length of root (cm) with IBA at 5 WAI

	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability
Between	6	234.626	39.104	596.362	0.0
Within	28	1.836	0.066		
Total	34				
CV%	3.65				
LSD value	0.3328				

Appendix XXXIII. Analysis of variance on average length of root (cm) with NAA at 5 WAI

	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability
Between	6	131.971	21.995	681.269	0.0
Within	28	0.904	0.032		
Total	34	132.875			
CV%	3.75				
LSD value	0.2318				

Appendix XXXIV. Analysis of variance on diameter of root with IBA at 5 WAI

	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability
Between	6	2.316	0.386	92.546	0.0
Within	28	0.117	0.004		
Total	34	2.432			
CV%	6.11				
LSD value	0.08194				

Appendix XXXV. Analysis of variance on diameter of root with NAA at 5 WAI

	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability
Between	6	75.982	12.664	428.279	0.0
Within	28	0.828	0.03		
Total	34	76.809			
CV%	4.82				
LSD value	0.2244				