CALLUS INDUCTION AND MICROPROPAGATION OF PINEAPPLE (*Ananas comosus* L.)

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CERTIFICATE

This is to certify that thesis entitled, **"CALLUS INDUCTION AND MICROPROPAGATION OF PINEAPPLE (*Ananas comosus* 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* **FATEMA AKTER,** *Registration No.* **09-03345** *under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.*

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

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DEDICATED TO MY HUSBAND

ABBREVIATIONS ANDACRONYMS

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
g/L	:	Gram per litre
BAP	:	6-BenzylAminoPurine
BA	:	Benzyladenine
KIN	:	Kinetine
IAA	:	Indoleacetic acid
IBA	:	Indolebutyric acid
NAA	:	*a*-Napthalene aceticacid
2, 4-D	:	2,4- Dichloro phenoxy acetic acid
Int.	:	International
J.	:	Journal
Mol.	:	Molecular
mg/L	:	Milligram per litre
µM	:	Micromole
MS	:	Murashige and Skoog
PGRs	:	Plant Growth Regulators
Res.	:	Research
Sci.	:	Science
CV	:	Co-efficient of Variation
^{o}C	:	Degree Celsius
etc.	:	Etcetera
WAI	:	Weeks After Inoculation

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The Author

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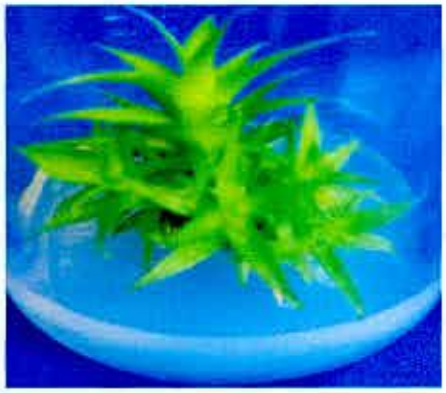
CALLUS INDUCTION AND MICROPROPAGATION OF PINEAPPLE (*Ananas comosus* L.)

BY

FATEMA AKTER

ABSTRACT

The present study was undertaken in Biotechnology Laboratory of the Department of Biotechnology, Sher-e-Bangla Agricultural University, Sher-e-Bangla Nagar, Dhaka-1207 during the period from January, 2014 to December, 2014 to evaluate the effect of different plant growth regulators on callus induction, shoot proliferation, root formation and to develop micropropagation protocol of pineapple. Crown tip of pineapple fruits were used as explants and inoculated in MS media supplemented with different conc. of Benzyl adenine (BA), Kinetine (KIN), Indolebutyric acid (IBA), Naphthaleneacetic acid (NAA) alone or in combination with each other. The treatment 3.0 mg/L KIN + 2.5 mg/L NAA showed the highest percentage (80%) of callus induction. But minimum days (21.20) to callus induction and maximum weight of callus (2.80 gm) was observed with 2.0 mg/L KIN + 1.5 mg/L NAA treatment. The highest 85% shoot induction was obtained with 3.0 mg/L KIN +1.0 mg/L NAA treatment and minimum 7.60 days was recorded with the same treatment. The highest number of shoots (18.40) after 6 weeks of subculture was recorded in 3.0 mg/L KIN + 1.0 mg/L IBA and the longest shoot (9.04 cm) was found with 3.0 mg/L KIN+2.0 mg/L IBA. The treatment 2.0 mg/L IBA+ 1.5 mg/L NAA showed the highest percentage (75%) of root induction, minimum 16 days to root induction and the longest root (7 cm). But the highest number of roots (15.60) per explants was recorded with 3.0 mg/L IBA+ 1.0 mg/L NAA after 6 weeks of subculture to rooting media. Regenerated plantlets showed, 85% survival in growth chamber conditions and 82% in shade house stage of hardening with Sand : Soil : Cow-dung (1:1:1) and 78 % in open atmosphere at direct sunlight.



Chapter I
Introduction

CHAPTER I

INTRODUCTION

The pineapple plant (*Ananas comosus* L.) belongs to bromeliad family (Bromeliaceae), which contains 50 genera and about 2500 known species (Duval *et al.*, 2003). The original name of the fruit comes from the word Tupi *nanas*, meaning "excellent fruit. The plant is indigenous to South America and is said to originate from the area between southern Brazil and Paraguay. However, little is known about the origin of the domesticated pineapple. It is cultivated in Thailand, Brazil, Costarica, Philipine, Indonesia and Brazil. Costarica, Brazil and Philipine are world top pineapple producing countries and they produce 2685131 tons, 2483831 tons, 2458240 tons respectively (FAOSTAT, 2013).

Pineapple (*Ananas comosus* L.) is one of the most economically important tropical fruits (Duval *et al.*, 2003). In terms of worldwide production, it is currently the third most important tropical fruit after bananas and mangoes. Among all the fruits produced in the country, pineapple ranks 4th in terms of total cropping area and production. According to year book of agricultural statistics of Bangladesh-2012. BBS, total area under cultivation was 34.5 thousand acres, total yield was 180.5 thousand metric tons and yield per acre was 5.2 metric ton. Bangladesh shares only 0.8% pineapple in the world pineapple market. So, Bangladesh should improve pineapple production to increase share in the world market.

The climate and the soil of many parts of Bangladesh are much more suitable for pineapple production. It tolerates neither very high temperature nor frost. Pineapple usually flowers from February to April and the fruits are ready from July to September. Sometimes, off-season flowers appear and they produce fruits in October-December. Pineapple should be grown in well-drained soils and areas of the landscape that do not flood. In Bangladesh Sylhet, Madhupur, Rangamati, khagrachari, Bandarban, Chittagong hill tract, Tangail, Dhaka and Ghorasal are the major pineapple producing area. Three varieties of pineapple are mostly grown in Bangladesh. Those are: Giant Kew, Honey Queen and Ghorasal.

Generally, the ripen pineapple is consumed by the people of Bangladesh. The fruit is utilized in the processing and canning industries. Pineapple is a great source of fiber and is high in vitamins B1& C. It also has B2, B3, B5, B6, beta-carotene, folic acid, potassium, magnesium, iron & copper. Pineapples are highly rich in the enzyme Bromelain which is great to eat as a digestion aid and also pineapple juice has an anti-inflammatory effect. Two 3"diameters, 3/4" slices of fresh pineapple contains 60 calories, 2g fiber, and are fat free. Green pineapple is also used for making pickles. After extraction of its juice, the left over is used as livestock feed and also the tender leaves are used for the same purpose. Various food items like squash, syrup, jelly, etc. are produced from pineapple. Vinegar, alcohol, citric acid, calcium citrate etc are also produced from it. Pineapple is also recommended as medical diet for certain diseased persons.

The pineapple is routinely propagated by means of lateral shoots, basal suckers or crowns, slip, gill. Growth is best with suckers and slips. Plants from crown bear flowers after 3 to 20 months later than suckers and slips depending on the climatic conditions. Therefore, crowns are not normally used.

This conventional method of propagation is slow and allows for transfer of pineapple requires large volume of planting materials, which are hardly obtained by conventional method of propagation. Vegetative propagation of pineapple from suckers and crowns is often restricted in some cultivars by the limited availability of propagules (Popluechai, *et al.* 2007). The major problems that affect the commercial production of pineapple are lack of high quality propagules, low rate of multiplication of plants and the lack of matrix plants. Fusarium wilt is another major problem of pineapple. The need to solve these problems, producing better and clean propagules, improving the rate of plant multiplication and a faster multiplication of elite genotypes, led to the development of tissue culture techniques for the pineapple (Almeida, 1994).

Plant tissue culture offers an opportunity for large scale production of uniform pineapple planting material in a relatively short period of time. Pineapple conventional technique can be considered to be easy, but the multiplication rate is low

and it would take eight years to obtain enough propagules from one mother plant (Almeiet *al.*, 2002). Being a vegetative propagated plant, conventional hybridization techniques for the generation of better pineapple varieties are cumbersome and time consuming (Mahatre, 2007).

Hence, the need to improve the multiplication rates of selected elite genotypes led to the development of tissue culture techniques for the pineapple. *In vitro* micro-propagation of pineapple plantlets has many advantages over conventional methods of vegetative propagation. This technique allows an efficient and rapid increase of selected elite pineapple varieties. According to Drew (1980), it is possible to produce 1,250,000 plantlets of pineapple in eight months, starting with 30 explants.

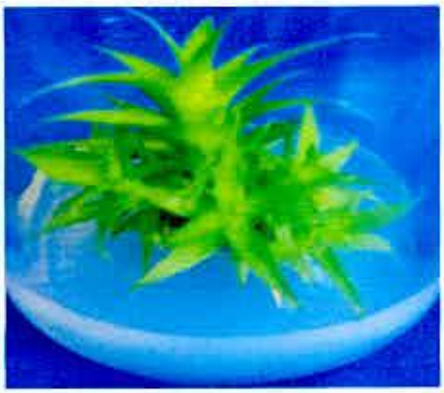
The plant hormones auxin and cytokinin are critical for plant regeneration in tissue culture, with cytokinin playing an instrumental role in shoot organogenesis (Hill and Schaller, 2013). Cytokinin in the culture medium plays important role for callus induction and shoot proliferation and auxin is necessary for rooting. Direct micro-propagation or tissue culture of shoot tips or crown has been successfully carried out in pineapple (Hammad and Taha, 2008). But still efficient regeration protocols in Pineapple are requisite to develop a rapid, efficient and easy method of micropropagation.

In this study, a new method of micropropagation of pineapple is being intended to develop using crown as explants through callus culture. This experiment emphasizes in pineapple tissue on the influence of Benzyladenine (BA), Kinetine (KIN), Indolebutyric acid (IBA) and Nephthaleneacetic acid (NAA).

OBJECTIVES:

The research proposal is designed with the following objectives-

- To develop *in vitro* regeneration protocol of *Ananas spp.*
- To study the hormonal effect on micropropagation in pineapple.
- To identify the best plant growth regulator for *in vitro* regeneration in pineapple.
- To study the acclimatization potentiality of *in vitro* regenerated plantlet under natural condition.



Chapter II
Review of literature

CHAPTER II

REVIEW OF LITERATURE

In vitro propagation of pineapples for plantlet regeneration and conservation is well documented. It has comparative advantage over the traditional methods as it leads to the production of large number of disease-free uniform planting materials in a shorter period independent of the season. The success of plant tissue culture depending very much on the stage and type of explants selection, the sterilization method and the type of culture media evident in many studies have been reviewed in this chapter.

2.1 Explants

Various types of explants can be used for tissue culture. Several parts of pineapple plant for micropropagation i.e crown, axillary bud of sucker, slip and gill, anther etc. Micropopagation of shoot tips and bud from crown have been reported for pineapple. (Al-saif *et al.*, 2011; Hammad and Taha, 2008; Kiss *et al.*, 1995). Sucker was suggested by Nelson *et al.*, 2012; Slips by Almida *et al.*, 2002 and Axillary bud by Vesko *et al.*, 2000.

Wald *et al.* (1988) reported increased multiplication rate of Smooth Cayenne by 40- to 85- fold over a period of 13 months by culturing crown meristem explants.

2.2 Sterilization of explants

Plant tissue culture media provides a good nutrient source for bacteria and fungi, therefore precautions against microbial contamination must be taken in all *in vitro* procedures.

Almida *et al.* (2002) sterilized pineapple slips which consisted of axillary buds obtained after careful excision of the leaves, in the hood with ethanol (70%) for three minutes followed by calcium hypochlorite (2%) for fifteen minutes and three washes in sterile water.

Dutta *et al.* (2013) developed a cost effective protocol of pineapple and stated that shoot apical meristems were first washed in tap water for at least 15 minutes followed by stirring with 20% Bavistin (20 minutes). After that, the explants were disinfected with Tween-20 and 0.5% sodium hypochlorite solution (15 minutes) followed by five times washing in CA water (solution of 0.25% citric acid and 0.5% ascorbic acid).

Khan *et al.* (2004) conducted a study on large scale multiplication of pineapple and developed a method of sterilization of explants. The stems were cut into transverse sections containing one nodal bud. They were cut into approximately 2cm long segments and used as explants for shoot initiation under *in vitro* conditions explants were sterilized using 1% Mercuric chloride and it was determined that a more effective method of sterilization would be soaking the plants in 0.1% Mercuric chloride for 3-4 minutes followed by rinsing the plants thoroughly in sterile distilled water for 5 minutes each to remove traces of mercuric chloride.

Nelson *et al.* (2015) conducted an experiment on *in vitro* growth and multiplication of Pineapple under different duration of sterilization. He found that NaOCl at 2% is effective for the sterilization of pineapple explants when the plant material is sterilized for 20 min beyond which the cells die to affect regeneration.

Pineapple crown were sterilized with 50% Clorox [sodium hypochlorite 5.2% (15 min)], 20% Clorox (10 min) and rinsed once with sterile distilled water by Zuraida *et al.* (2011)

2.3 Plant Growth Regulators

Ibrahim *et.al* (2013) conducted an experiment to test cytokinin type and concentration, and source of explants on shoot multiplication of pineapple plant (*Ananas comosus* 'Queen') *in vitro.* The results showed that MS medium supplemented with 1.0 mg/L BA and 0.2 mg/L NAA gave adventitious shoots directly after two months from culturing of callus. Callus and nodal segments cultured in MS medium supplemented with 1.0 mg/L KIN was significantly superior on other treatments studied (0.5 mg/l KIN, 0.5and 1.0 mg/L BA) in number of shoots/explants,

leaf length, number of leaves/shoot and leaf area of formation shoot which reached 18.60 shoot/explant, 5.38 cm, 10.60 leaves/shoot and 3.64 cm^2, respectively.

Farahani (2014) focused over the mass production of high quality plantlets of pineapple. He showed that higher multiplication rates for *Ananas comosus* L. were obtained with BA concentrations of 5mg/L at 3 months. The *in vitro* proliferated shoots produced roots with maximum frequency (84%) on MS medium without growth regulator at 6 weeks intervals. Moreover he found that it is possible to obtain 1 million rooted plantlets after 12 months from a single bud, with a 45 day subculture interval.

Usman *et al.* (2013) developed an efficient protocol for micropropagation of pineapple. He used axillary bud from crown and found that MS supplemented with BA (5 µM) and NAA (3 µM) gave the highest number of plantlets of 11.5 and 14.4 and the highest mean plant height at shoot elongation of 5.8 and 7.6 cm, respectively.

Amin *et al.* (2005) carried out an experiment for large scale pineapple production through leaf derived callus culture .He found that the cultured explants produced callus within 4 weeks of incubation on media supplemented with 0.5-3.0 mgL-1 2,4-D alone and in combination with 0.5-3.0 mg/L BA. Maximum number of shoot buds with optimum callus growth was observed on MS medium containing 1.0 mgL-1 BA and 0.1 mg/L NAA after six weeks of culture. Rooting performance was best on MS medium containing 0.2 mg/L IBA+0.2 mg/L NAA.

Elsevier and Debergh (2006) developed a low cost micropropagation procedure for pineapple (*Ananas comosus* L.). He fouud that MS medium, supplemented with 0.8–1.0 mg/L BA of medium yielded the best multiplication, with about 8 to 9 shoots during a culture interval of 8 weeks.

Awal *et al.* (2010) used pineapple crown tip meristem as explants for pineapple micropropagation. The explants were maintained in 16 hours light 8 hours dark, at room temperature 25±2°C. Optimum regeneration was obtained onto MS supplemented with 3.0 mg/L and MS containing 1 mgL-1 BAP / 0.5mg/L NAA.

Zuraida *et.al* (2011) established a novel approach for rapid micropropagation of pineapple shoot using liquid shake culture system. Explants were subsequently sub-cultured on medium with 1 mg/L BAP which produced highest number of proliferated in vitro plantlets. The optimization of the conditions for shoot propagation was carried out in both liquid and solid medium by supplementing with 1 or 5 mg/L of BAP. MS liquid medium supplemented with 1 mg/L BAP produced the highest number of shoots (31) after 4 weeks. The number of shoots formed was increased to 204 after third sub-culture in liquid medium.

Al-Saif *et al.* (2011) observed effects of benzylaminopurine and naphthalene acetic acid on proliferation and shoot growth of pineapple (*Ananas comosus* L. Merr) *in vitro.* BAP at 2.0 mg/L significantly affected the production of shoots per explant, shoot length and weight. Total shoot length was higher in BAP (2 mg/L) than in control (MS medium without hormone) and NAA (0.2mg/L) after 10, 20, 30, 40, 50 and 60 days incubation period. Total shoot length was highest in BAP in all incubation periods. Total shoot weight was higher in BAP (2 mg/L) and lower in NAA (0.2 mg/L) as compared to MS medium without hormone.

Nualbunruang and Chidburee (2004) researched on the effect of different type and concentrations of auxin on *in vitro* rooting and the effect of different growing media on the growth of two hybrid pineapples (Smooth cayenne and Queen. Results showed that after 12 week cultivation, the MS supplemented with NAA at 2 mg/L gave the highest number of roots, 33.4 roots per shoot in the Smooth cayenne variety and MS supplemented with NAA at 2 mg/L gave the highest number of roots, 24.8 roots per shoot in the Queen variety.

Akbar *et al.* (2003) cultured meristem tips of the crown of *Ananas comosus,* on MS supplemented with 1.5 mg/L NAA and 1.0 mg/l Kn, callused within three weeks. This callus, when subcultured on MS with 1.5 mg/L KIN + 0.5 mg/L NAA produced a large number of shoots. He found that Shoots rooted well within two weeks, when they were excised individually and implanted in half strength MS with 2.0 mg/l IBA. Eighty percent plantlets survived when transferred to open field.

Khan *et al.* (2004) carried out an experiment for the micropropagation of pineapple. BAP @ 0.5 mg/L showed better results for the number and length of shoots per explant. IBA and NAA were employed for appropriate root initiation and development and the best media observed containing IBA @ 1.5 mg/L alone.

Roy *et al.* (2000) developed a protocol of mass propagation of Pineapple through *in Vitro* Culture. Meristem tip of crown were cultured on MS medium supplemented with 1.5 mg/L NAA and 1.0 mg/L kinetin callus was initiated within 3 weeks. This callus, when subcultured on MS medium with 1.5 mg/L kinetin + 0.5 mg/L NAA, produced large number of shoots. He found that shoots were rooted well within two weeks, when they were excised individually and implanted in half strength MS medium with 2 mg/L IBA.

Vesco *et al.* (2001) worked on improving pineapple micropropagation protocol through explant size and medium composition manipulation. Axillary buds were inoculated on an MS liquid culture medium added with NAA (2 µM) and BAP (4 µM).He found that the highest multiplication rate (13.5 shoots) was obtained with the smallest shoots inoculated, while in the MS culture medium free of growth regulators, the highest plantlets (7.7 cm) were the result of the highest shoots inoculated and showed no vitrification. For acclimatization, the highest values of the survival rate (93.8%) and fresh and dry weights were obtained with the transference of higher than 7.0 cm *in vitro* plantlets.

Jiaxie *et al.* (2005) emphasized on tissue culture and rapid propagation with low cost of pineapple cultivar Mibao. He concluded that the appropriate medium for germination of adventitious buds is MS + 3mg/L BA + 0.1 mg/L NAA. When MS + 4.0 mg/L BA + 0.1 mg/L NAA is used as multiplication medium, the multiplication rate could be increased by 8.1 times after 30 days cultured; using static liquid culture in this medium without adding agar, it could amplify 18.2 times. The best rooting medium was 1/2 MS + 3.0 mg/L IBA + 0.5 mg/L NAA. When the plantlets were transplanted to red loam + sand + organic fertilizer (3:1:1), the survival rate could reach 95% and the plants grew well.

Danso (2008) worked on improvement of multiplication rate of MD2 pineapple planting material. He found that liquid cultures required 5.0 mg/L BAP to significantly ($P<0.05$) increase the multiplication rate by 2- or 5-fold compared to 7.5 mg/L in solid cultures. Moreover, when plantlets from optimal treatments were transferred to liquid medium with high concentration (7.5-15 mg/L) of NAA there was no root development. The number of root produced per plantlet on a medium supplemented with a combination of NAA and IBA was comparably higher than when NAA or IBA alone were used.

Wasaka *et al.* (1978) cultured various parts of *Ananas comosus* (young syncarp, anther, auxiliary bud of sucker or slip, small crown and small slip on MS media containing benzyladenine (BA) and 1 –naphthalene acetic acid (NAA). Except anther all parts produced nodular or globular body different from so-called callus. He found that globular and nodular body grew vigorously and induced numerous plant on 2mg/L BA and NAA. Their ability does not decrease after several sub-culture and showed varied characteristics.

Hamad and Taha (2008) tested *in vitro* mass propagation using the direct and indirect shoot proliferation techniques. The plantlets were successfully initiated from sucker on solid MS basal medium containing 30 g/L sucrose, 0.1 g/L Myo-inositol and 3 mg/L BAP after one month of culture. The highest direct shoot tips regeneration was obtained on solid MS medium when added with 30 g/L sucrose, 0.1 g/L Myoinositol, 3 mg/L BAP and 1 mg/L NAA. Indirect shoot regeneration was obtained on medium containing 3 mg/L Zeatin after one month of culture. In average, 10 shoots were regenerated from approximately 1 gram of calli. The techniques can produce 100-200 number of plantlet within 4 to 6 months of culture, and ready for planting after 7 months of culture.

Roostika and Mariska (2003) worked on *in vitro* Culture of Pineapple by Organogenesis and Somatic embryogenesis. An indirect technique was done by first producing nodular globular structures followed shoot regeneration. The highest level of regeneration by direct organogenesis was obtained on an MS medium containing 27 µM NAA and 1 µM BA, with a 99% regeneration percentage while that by indirect organogenesis, on obtained from the same medium, was only 86% regeneration

percentage. The percentage of explants producing shoot and the average number of shoots per crown produced from the longitudinal section (100%) was higher than those from the leaf base (88%).

Akin-idowu *et al.* (2014) conducted a study on influence on medium type and growth regulator on *in vitro* regeneration of pineapple. They used shoot tip explants. They found that semi-liquid MS basal medium supplemented with 0.1mg/L and 0.3 mg/L BAP gave the highest regeneration result showing highest shoot length 34.6mm and average shoot buds 2.4 per explants. Significant difference in average shoot bud was observed with semi-liquid MS basal medium supplemented with BAP 1.5 mg/L produced highest average shoot no. 6.1 per explants after 5 weeks and increased upto167.6 after 20 weeks. The half strength MS basal with 0.9 mg/l NAA gave the highest root length 29.3 mm and root no. 7.9. They concluded that semi-liquid MS basal medium supplemented with low BAP 1.5 mg/L is a cost effective micro-propagation technique of pineapple.

Hamid *et al.* (2013) examined to identify the optimal combination for root formation using two cultivars of smooth cayenne and morris. All the shoots of both cultivars rooted in solid medium enriched with 2.0 mg/L IBA at quarter strength for Smooth Cayenne and half strength for Morris. Full strength solid medium enriched with 1.0 mg/L NAA was the best choice for rooting of both Smooth Cayenne and Morris, resulting in the tallest plantlets (74.1 mm), highest number of roots per shoot (11.4) and intermediate rooting percentage (89.5%).

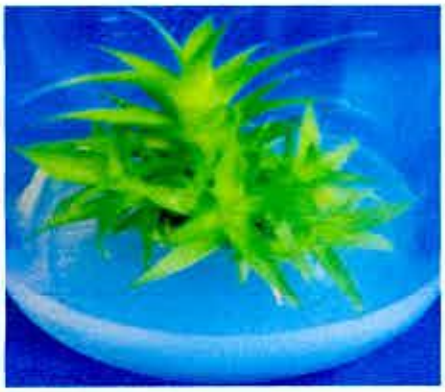
3.4 Acclimatization of regenerated plantlets

Farahani (2013) stated that for *ex vitro* hardening, plantlets were placed in pots containing 82 cm3 of a mixture pit moss + perlite (2:1) as acidic soil. During hardening of *in vitro* plantlets, they were transferred to the greenhouse environment (photon flux density of 458 µmolm–2s–1 and humidity 70% to 80%).

Nualbunruang and Chidburee (2004) used seven different media to observe significant impact on some selected parameters of vegetative growth of pineapples. In general, mixture of sand :coconut-compost :charcoal husk (1:1:1), sand :charcoal husk (1:1), soil :sand :charcoal husk (1:1:1) and soil :sand :charcoal husk (1:1:1) gave higher

values of growth parameter such as number of leaves per plant, plant height, leaf length and leaf width in comparison to other media ($p<0.05$). However, the medium prepared from the mixture of soil: sand (1:1) gave the highest shoot per explants. Considering the physical characteristics, aeration and water holding capacity are probably the most important factors while chemical characteristics, e.g. nutritional status and salinity level have a crucial role on plant development.

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



Chapter III
Materials and Methods

CHAPTER III

MATERIALS AND METHODS

3.1 Time and location of the experiment

The present research was carried out in Biotechnology Laboratory of the Department of Biotechnology, Sher-e-Bangla Agricultural University, Sher-e-Bangla Nagar, Dhaka-1207 from the period of January, 2014 to December, 2014. Four sub-experiments were conducted to fulfill the objectives of the present study.

Sub-Experiment 1: Callus induction from pineapple crown

Sub-Experiment 2: Multiple shoot regeneration in pineapple

Sub-Experiment 3: Root induction in pineapple

Experiment 4: Acclimatization of regenerated plantlets of pineapple

3.2 Experimental materials

3.2.1 Source of material

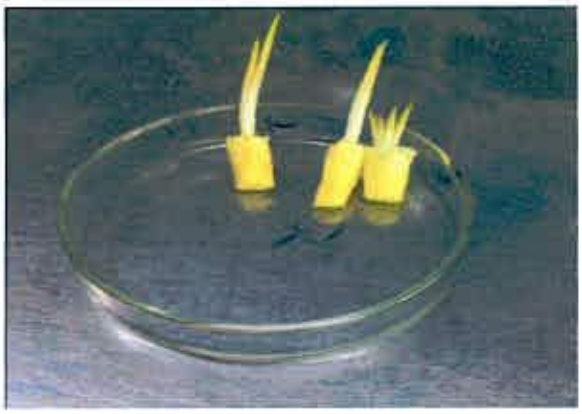
The planting materials of pineapple (*Ananus comosus*) were collected from nursery nearby Agargaon area, Sher-e-Bangla Nager, Dhaka-1207.

3.2.2 Plant materials

The fresh pineapple fruits with bright green crowns were used for the experiment. Fresh, healthy and disease free crown were collected in a beaker filled. The explants were washed thoroughly with running tap water for removing soil. Crown with young leaves were collected from fruits showing good biomass yield (Plate 1A, 1B). The extra leaves were removed and shoots were trimmed to size of 1.5-2 cm for further work (Plate 1C).

1A

1B



1C

Plate 1. Plant material of *Ananas comosus* for micropropagation

3.2.3 Instruments and glass ware

Metal instruments *viz.*, forceps, scalpels, needless, spatulas and aluminum foils were used.The Borosil glasswares were used for all the experiments. Oven dried (250^0C) Erlen 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.

3.2.3 Culture medium

The most important factor of success in tissue culture is mainly related to the choice of nutritional components and growth regulators. Presence of plant growth regulators plays a significant role in a successful regeneration of any plant species. Media for tissue culture should contain all major and minor elements, vitamins and growth regulators which are essential for normal plant growth. Explants were inoculated onto media composed of basal MS (Murashige and Skoog, 1962) medium supplemented with the plant growth regulators. Hormones were added separately to different media according to the requirements. The phytohormone and their concentrations were given below-

- BA (1.0, 2.0, 3.0, 4.0 mg/L) alone and in combination with NAA (1.0, 2.0, 3.0 and 4.0 mg/L), KIN (1.0, 2.0, 3.0, 4.0 mg/L) alone and in combination with IBA (1.0, 2.0, 3.0 and 4.0 mg/L) and NAA (1.0, 1.5, 2.0) were used for shoot proliferation.
- NAA (1.0, 1.5, 2.0, 2.5 and 3.0 mg/L) and IBA (1.0, 1.5, 2.0, 2.5 and 3.0 mg/L) were applied separately and in combination both of them for root formation.
- Sucrose (3%) was used as carbon source and media were solidified with agar (0.8%).The pH was adjusted to 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

MS medium is prepared by the combination of stock solutions with different minerals and hormones required for plant regeneration and growth. Each stock solution is composed of different types and amount of major salt, minor salts, iron and organic,

growth regulators etc respectively. All the chemicals used for stock solution is highly purified and labeled as plant tissue culture tested grade. The chemicals are dissolved in double distilled water or highly purified de-ionized water. Each chemical are added according to the list of ingredient presented in Appendix-1

3.3.1 Preparation of stock I solution

Stock solution of macro nutrients or stock I was prepared with 10 times (10x) of the final strength of the medium in 1000ml of distilled water. Salts are weighted accurately and dissolved completely 10 times the weight of the salts required for one litre of medium in 750 ml of distilled water. Each chemical was completely dissolved before adding other chemicals. The final volume was made up to one litre by adding distilled water. The stock solution was filtered through what man no.1 filter paper to remove all the solid particles. The stock I solution was stored in a glass container, tagged with date of preparation and stored in refrigerator at 4 ± 1^{0}C.

3.3.2 Preparation of stock II solution

A stock solution of all micronutrients with 100x concentration is generally prepared. Since copper and cobalt are required in very small quantities, it was prepared in a separate stock solution and pipetted into main micronutrient stock solution. This stock solution was also stored in refrigerator at $4\ ^{0}$C.

3.3.3 Preparation of stock III solution

The stock III solution is the solution of iron-EDTA which was added freshly and made hundred folds (100x) the final strength of the medium in 1000ml of solution. FeSO4 and Na-EDTA was dissolved in 750 ml of distilled water in a conical flask by heating in a water bath until the salts dissolved completely and final volume was made upto one litre by addition of distilled water. The stock was stored in an amber color bottle and kept in refrigerator at $4\ ^{0}$C.

3.3.4 Preparation of stock IV solution

The stock IV solution is composed of vitamins and amino acids prepared by adding pyridoxine HCL (Vitamin B6), Thiamine HCL (Vitamins B2), Myo-inositol (insitol),

Glysine and Nicotine acid (Vitamin B3). Each of the vitamins except myo-inositol was taken at 100 times of their final strength in measuring cylinder and dissolved in 400 ml of distilled water. The final volume was made upto 1000 ml by adding further distilled water. But the myo-inositol was made separately 100 folds the final strength of the medium 1000ml distilled water. Then the stock was stored in refrigerator at 4 ± 1^{0}C after filtration.

3.3.5 Preparation of hormones and growth regulator

Auxin and Cytokinin are growth regulator used for plant growth development supplemented with MS medium. Solubility of such hormones are different. They are found in powder form in the market. That's why we need to make stock solution of them. In the experiment four types of growth regulators are used. They were Kinetin (KIN), Benzyladenine (BA), Indole -3 – butaric acid (IBA), 1-Naphthaleneacetic acid (NAA). Molecular weight and solubility of these hormones are shown in appendix II. To prepare 100ml stock solution of hormones, 100mg powder of hormone was added and dissolved it with their respective solvents in color amber bottles. Finally the volume was made 100ml by adding distilled water. Concentration of compounds was taken as mg/L. Then it was stored in refrigerator at $4\ ^{0}$C.

3.3.6 Preparation of other stock solution

3.3.6.1 Preparation of 1N NaOH

40 gm of NaOH pellets were dissolved in one litre of distilled water to prepare 1N NaOH stock solution. Prepared solution was stored in cool and dry places in a glass bottle. This solution was used for adjusting pH of the final culture media preparation.

3.3.6.2 Preparation of 1N HCl

To prepare 1L of 1 N solution of HCl, 36.5 g of the substances was dissolved in 1 L of water.It was used for adjusting pH of the cultural medium to decrease pH meter reading.

3.3.6.3 Preparation of 70% ethanol

In a 100 ml measuring cylinder 70 ml 99.9% ethanol was poured. Then 30ml double distilled water was added to make 100ml 70% ethanol. It was stored in sterilized glass bottle. This solution was made fresh each time before use.

3.3.6.4 Preparation of 10% NaOCl

To prepare 100 ml NaOCl, 10 gm of NaOCl powder was poured in a beaker and added distilled water upto make final volume. Then the solution was kept in Hot Plate magnetic Stirer to dissolve it completely. Then it was cooled in refrigerator at 4^0C.

3.3.6.5 Preparation of 5% NaOCl

To prepare 5% NaOCl, 5 gm NaOCl was dissolved in 100ml distilled water.

3.4 Preparation of culture media from MS stock solution

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

Step-1. 500 ml of sterile distilled water was poured into 1000 ml beaker.

Step-2. 1-100ml MS stock solution -I, 10 ml MS stock solution-II, 10 ml stock solution –III, 10 ml MS stock solution-IV and 30 gm of sucrose was added and gently stirred to dissolve these ingredients completely with the help of a Hot Plate Magnetic Stirrer.

Step-3. Different concentrations of hormonal supplements (1, 2, 3, 4) mg/L 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 Sterilization

3.5.1 Sterilization of Glassware and instruments

All type of glassware and instruments were first rinsed with 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 autoclave at 121 ^{0}C for 15 psi for 30 minutes.

3.5.2 Sterilization of culture medium

For sterilization the culture medium was poured in 1000 ml culture bottles and then autoclaving was done at a temperature of 121^{0}C for 25 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 semi-solid for inoculation with explants.

3.5.3 Sterilization of culture room and transfer area

Before using culture room it was carefully cleansed with detergent and wiped with 70% ethanol. The room was sprayed with formalin by wearing protective mask to avoid risk. After spraying the whole room was concealed for 24 hours.

3.5.4 Sterilization of Laminar Air Flow Cabinet

The laminar air flow cabinet was started half an hour before working. The air flow cabinet surface was cleaned with cotton soaked with70% ethanol. All glassware was kept on the cabinet to reduce contamination except culture media. The lid of cabinet was closed well and UV was switched on for 30 minutes while turning off the air flow. After required time was over, UV was switched off, opened the door and switched on the air flow.

Within 5 minutes, work was started. The forearms and hands were sterilized by rubbing 70% ethanol before started working. During the culture all equipment were frequently flamed after dipping with 95% ethanol.

3.6 Culture method

3.6.1 Explants culture

3.6.1.a Sterilization of explants

The trimmed crown shoot tip were washed thoroughly with autoclaved distilled water for several times. Then the explants were transferred to laminar air flow cabinet and kept it sterilized beaker. Then it's were treated with 70% ethanol for 1 minute and rinsed with autoclave distilled water for 3 times. NaOCl was filtered by a sterilized filter. Then the explants were immersed in 10% NaOCl for 10 minutes with 1 drop of Tween-20 and rinsed 2 times with distilled water thoroughly. After then it was immersed with 5% NaOCl for 5 minutes and washed with 2 times with distilled water. The beaker with explants was constantly shaken during sterilization to make the material free from chemical and ready for inoculation.

3.6.1.b Inoculation of culture

The sterilized explants were inoculated carefully following proper sterilization process within laminar airflow cabinet. Prior to use, the surface of the laminar flow bench was swabbed down with 70 % ethyl alcohol and the interior sprayed with the same alcohol. All glassware, instruments and media were steam-sterilized in an autoclave. During the course of the work, 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 explants on the medium.

For inoculation, explants were transferred to large sterile glass petridish or glass plate with the help of sterile forceps under strict aseptic conditions. Here the explants were further trimmed and extra outer leaves were removed with sterile scalpel blade to make suitable size. After cutting explants into suitable size (1.5-2.0cm), explants are transferred to culture bottles containing MS medium with plant growth regulator (Plate 2). Each bottle was contained 20 ml of culture medium. After vertically inoculating the explants singly in culture bottle, the mouth of bottle was quickly

flamed and capped tightly. After proper labeling, mentioning media code, date of inoculation etc. the bottles was transferred to growth room.

Plate 2. Inoculation of *Ananas comosus* in culture medium

3.6.1.c Incubation of culture

The prepared cultures were kept in a growth room on the shelves. All the cultures were kept at 25±1^{0}C with a photoperiod of 14 hour daily with a light intensity of 3000 lux. After 15 days of inoculation the explants were turned whitish to dark greenish (Plate 3).

Plate 3. Incubation of inoculated culture vial

3.6.2 Subculture

3.6.2.1 Subculture of the callus for shoot regeneration

After six weeks of incubation, the calli attained suitable size for transfer to shoot induction media. Callus were removed aseptically from the vial in the laminar air flow cabinet and put on the sterilized petridishes. Then they were cut into small pieces and subcultured into the vial containing 25 ml shooting hormone in each. For shoot induction and development, KIN and BA alone or in combination with NAA, IBA were used. The cultured vials were kept in 16 hour photoperiod at 25±2 ^{0}c. Observation of explants growth and data collection were noted regularly. Contaminated vial were discarded carefully from culture room.

3.6.2.2 Subculture of the regenerated shoot for root induction

After 6 weeks of proper development, shoot grew about 4-5 cm in length were excised from the culture vial and transferred to root induction media aseptically in the laminar air flow cabinet. Rooting hormone IBA and NAA alone or in combination of them were used. Data was recorded after 3 and 6 weeks of subculture.

3.7 Acclimatization

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

3.7.1 Transfer of rooted plantlet to *in vivo* condition

For hardening, 42 days old well development, fully matured rooted plants were removed from the culture vial with the help of forceps. Then the plantlets were washed thoroughly in the running tap water to remove adhering culture medium. The plantlets were transferred to plastic pots containing autoclaved garden soil, sand and cow-dung in the ratio of 1:1:1 and kept in culture room for hardening after covered with plastic paper. In this time, regular irrigation was done. After 7 days, the plants were shifted to shade house. Initially plants were kept in high humidity and low light intensity. Later humidity was increased gradually to adapt the open environment.

3.7.2 Transfer of plantlets to the open field

After 15 days of hardening, the well adapted plantlets were transferred to the pot of bigger size. Finally, the plantlets were too sustainable and transferred to the open filed after 1 month.

3.8 Experimental Design

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.

3.9 Calculation of Data

To investigate the effect of different treatment of this experiment, the following parameters were recorded.

3.9.1 Callus color and texture

Callus color was observed and recorded for further study. Callus texture was measured by either friable or non-friable/compact for their physical characteristics.

3.9.2 Days to callus induction

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

3.9.3 Calculation of percentage of callus induction

Percent of callus induction were noted after 21 days of inoculation by using following formula:

$$\text{Percent (\%) of callus induction} = \frac{\text{No. of explants induced calli}}{\text{No. of explants incubated}} \times 100$$

3.9.4 Fresh weight of callus (gm):

Callus weight was recorded after 3 and 6 weeks of inoculation of explants with the help of electrical balance inside the laminar airflow with proper precaution. After that the callus was transferred to shoot induction media.

3.9.5 Calculation of days to shoot and root induction

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

3.9.6 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:

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

The percentage of root induction was calculated as:

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

3.9.7 Calculation of number of shoot and root per explants

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

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

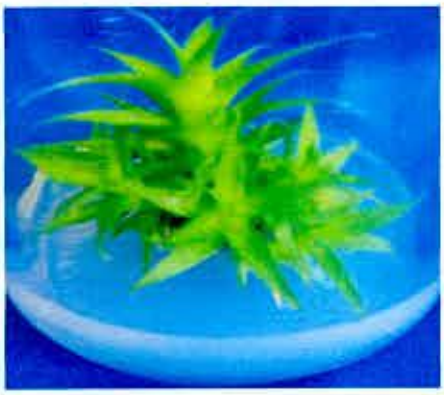
3.9.8 Calculation of shoot and root length (cm):

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

3.9.9 Calculation of survival rate of plantlets:

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

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



Chapter IV
Results and Discussion

CHAPTER IV

RESULTS AND DISCUSSION

Regeneration of pineapple from crown offers unique facilities for development of efficient protocol with a view to supply virus and disease free planting material for large scale commercial cultivation. Four separate experiments were performed for the rapid micropropagation of pineapple. The overall objective of the present study was to develop a system for the mass propagation of pineapple. The results of the experiment have been presented and discussed in this chapter with Plates (4-15), Figures (1-16) and Tables (1-19). Analyses of variance in respect of all the parameters have been presented in Appendices (I-XLV).

4.1 Sub-Experiment 1: Callus induction from pineapple crown

Callus culture technique has advantages over direct regeneration from lateral shoot and crown culture. It is often used for genetic transformation. To develop profuse callus, auxin and cytokinin hormonal treatment was applied. Callus induction from the prepared pineapple crown was done on MS media with different level of KIN alone or in combination with NAA with a view to find optimum growth regulator and concentration. The results of the study have been presented and discussed under following headings.

4.1.1 Effect of KIN on callus induction

The results of the effect of different concentrations of KIN have been presented under following headings with Figure 1, Table 1- 2 and Plate 4.

4.1.1.1 Percent of explants showing callus induction

There was significant variation on percentage of explants showing callus induction at different concentrations of KIN. The highest percentage (55%) of callus was induced in the treatment 3.0 mg/L KIN and the lowest percentage (25%) was induced in hormone free media. (Table1). Akbar *et al.* (2003) reported the maximum 30% callus induction in 1.5 mg/L KIN and 15 % in 0.5 mg/L KIN. There was an increasing trend of callus induction of pineapple with increasing the concentration of KIN.

Table 1. Effect of KIN on percent of callus induction in pineapple

Name of phytohormone	Concentration (mg/L)	No. of explants inoculated	No. of explants initiated callus	Percent of explants showing callus initiation
Control	0.0	20	5	25
KIN	1.0	20	8	40
	2.0	20	9	45
	3.0	20	11	55
	4.0	20	10	50

4.1.1.2 Days to callus induction

Significant variations were observed among different concentration of KIN on days to callus induction. The maximum days to callus induction were recorded in control (45 days) and 3.0 mg/L required minimum 24.40 days (Figure 1).It has been observed that no significant variation on days to callus among 1.0 mg/L (27.40 days) and 4.0 mg/L (27.60 days) of KIN.

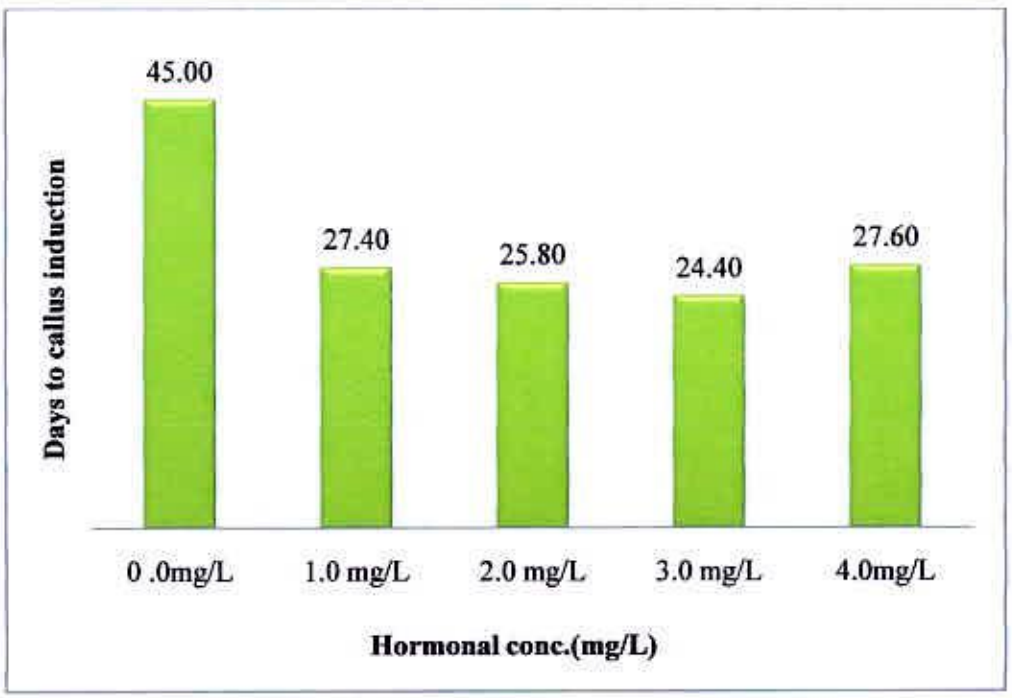


Fig.1. Effect of KIN on days to callus induction of pineapple

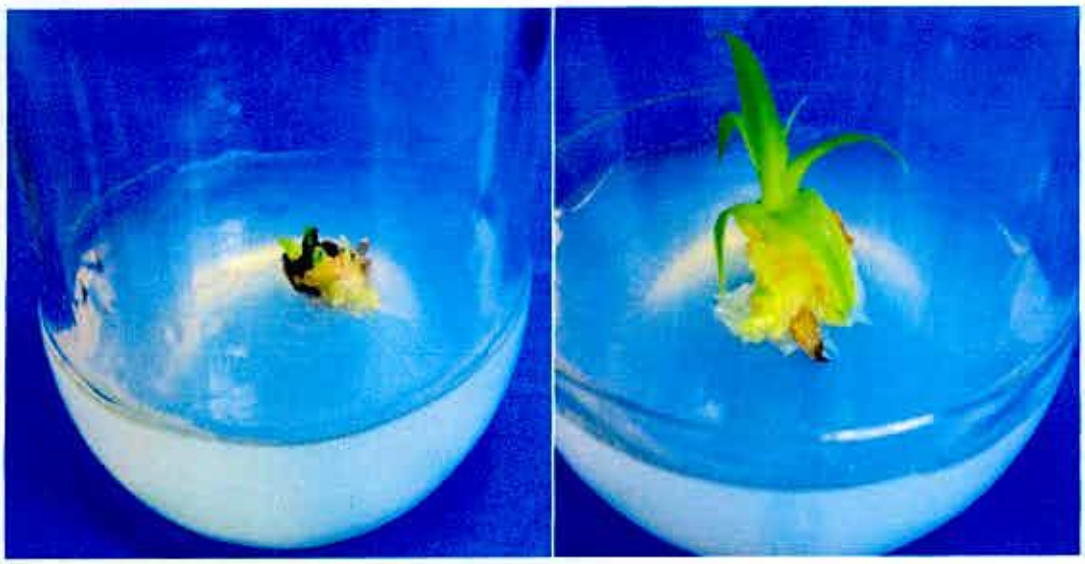
4.1.1.3 Fresh weight of callus (gm)

There was significant difference in initial and final weight of callus with the different concentration of KIN. The maximum weight of callus (1.44 gm) after 3 weeks was found in the treatment of 3.0 mg/L KIN whereas no callus was found in control after 3 weeks. After 6 weeks of inoculation, maximum weight was recorded (2.10 gm) at 3.0 mg/L KIN. On the other hand, lack of hormone produced minimum weight (0.88 gm) of callus. There was a little difference among 2.0 mg/L and 4.0 mg/L of KIN for weight of callus.

Table No. 2: Effect of KIN on weight of callus after 3 and 6 weeks of inoculation

Name of phytohormone	Concentration(mg/L)	Fresh weight of callus (gm)	
		3 WAI	6 WAI
KIN	0.0	0.00d	0.88d
KIN	1.0	1.22c	1.66c
KIN	2.0	1.36ab	1.84b
KIN	3.0	1.44a	2.10a
KIN	4.0	1.32b	1.94b
LSD (0.05)		0.0848	0.1341
CV (%)		5.62	5.83

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.



A B

Plate 4. Callus initiation on MS media supplemented with 3.0 mg/L KIN after (A) 3 weeks and (B) 6 weeks of inoculation

4.1.2 The combine effect of KIN + NAA on callus induction

The result of the combined effect of different concentrations of KIN + NAA have been presented under following headings with Tables 3-4 , Figure 2 and Plate 5.

4.1.2.1. Percent of explants showing callus induction

There was significant variation of KIN+NAA concentration on percent of explants showing callus induction. The highest percentage (80%) of callus was induced in treatment 3.0 mg/L KIN + 2.5 mg/L NAA and the lowest percentage (25.0%) was induced in hormone free media.

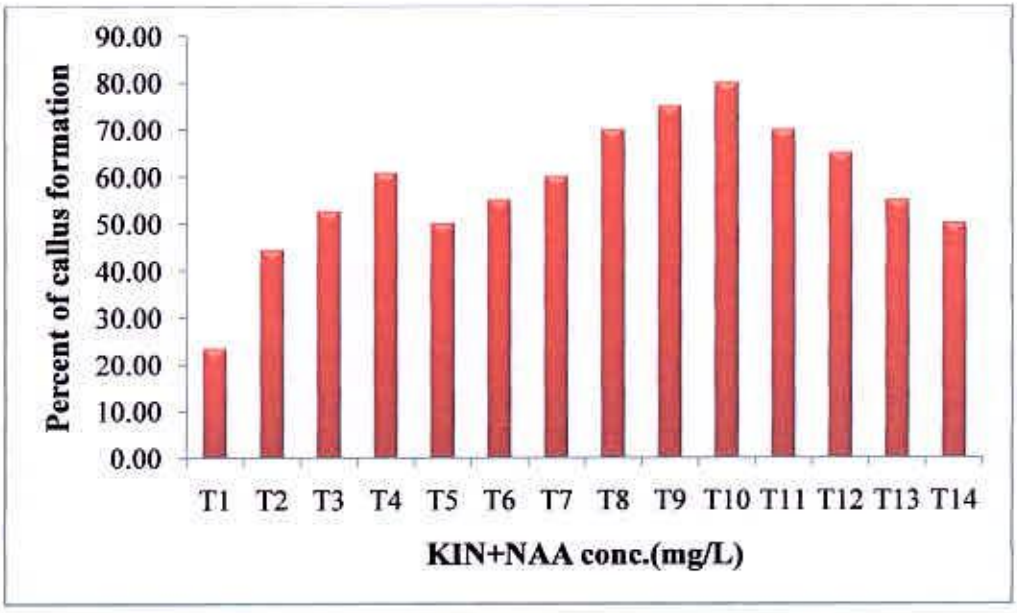


Fig.2. Combined effect of KIN+NAA on Percent of callus formation

Hormonal conc. (mg/L):

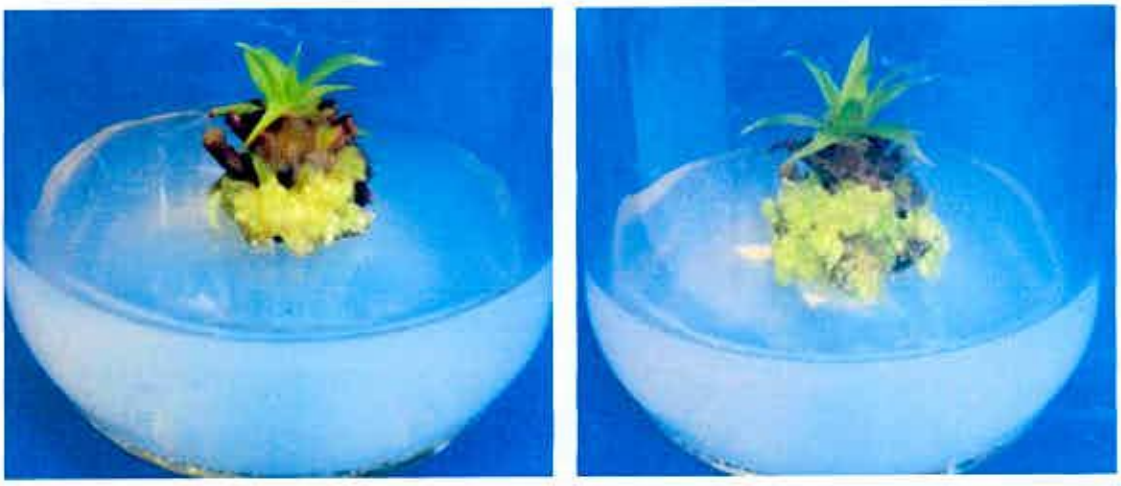
T1= NIL	T4=2.0+1.5	T7=3.0+1.0	T10=3.0+2.5	T13=4.0+2.0
T2=1.0+1.0	T5=2.0+2.0	T8=3.0+1.5	T11=4.0+1.0	T14=4.0+2.5
T3=2.0+1.0	T6=2.0+2.5	T9=3.0+2.0	T12=4.0+1.5	

4.1.2.2 Days to callus induction

Variations were observed among different concentrations of KIN+NAA on days to callus induction. The highest number of days to callus induction was recorded in control (45.0 days) and 2.0 mg/L KIN+ 1.5 mg/L NAA required lowest 21.20 days (Table 3).

4.1.2.3 Weight of callus

There was significant difference in initial and final weight of callus with the different concentration of KIN +NAA combination of hormone. The maximum weight of callus (2.08 gm) after 3 weeks was found in the treatment of 2.0 mg/L KIN+1.5 mg/L NAA combination. Whereas no callus was found in control after 3 weeks. After 6 weeks of inoculation, maximum weight was recorded (2.80 gm) at 2.0 mg/L KIN+ 1.5 mg/L NAA combination. On the other hand, lack of hormone produced minimum weight (0.88 gm) of callus (Plate 5 and Table 3).



A B

Plate 5. Callus induction on MS media supplemented with 2.0 mg/L kin+ 1.5 mg/L NAA after (A) 3 weeks and (B) 6 weeks of inoculation

Table 3. Combined effect of KIN +NAA on callus induction potentiality

KIN (mg/L)	NAA (mg/L)	Days to callus induction	Weight of callus (gm)	
			3 WAI	6WAI
MS-Control		45.00a	0.00j	0.88h
1.0	1.0	24.00cde	1.54g	1.94g
2.0	1.0	22.80ef	1.82cde	2.34c
	1.5	21.20g	2.08a	2.80a
	2.0	23.60bcde	1.98b	2.58b
	2.5	23.40def	1.90bc	2.24cd
3.0	1.0	22.40fg	1.74e	2.28c
	1.5	23.60def	1.86cd	2.60b
	2.0	24.20bcde	1.76de	2.32c
	2.5	22.20fg	1.64f	2.14de
4.0	1.0	24.60bcd	1.62fg	2.04efg
	1.5	24.60 bcd	1.54g	2.24cd
	2.0	25.60b	1.36h	2.12def
	2.5	25.40bc	1.32i	2.00fg
LSD (0.05)		1.381	0.08027	0.1204
CV (%)		4.5	4.21	4.4

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.

4.1.2.4 Comparative performance of KIN and KIN+NAA on callus induction in pineapple

The comparative performance of KIN alone or in combination with NAA used in present experiment revealed variation and it is necessary to find out the best hormone dose for each of the parameter under consideration. It has been observed that, KIN +NAA combination performed better than KIN alone in respect of percent of explants showing callus induction, days to callus induction and weight of callus. A total of 55.0% explants showed callus induction with 3.0 mg/L KIN , while 80% explants showed callus induction with 3.0 mg/L KIN +2.5 mg/L NAA. The minimum time (24.40 days) for callus induction was recorded in 3.0 mg/L KIN whereas it was 21.40 days in media containing 2.0 mg/L KIN + 1.5 mg/L NAA. It was found that weight of callus after 3 weeks with 3.0 mg/L KIN alone was 1.44 gm whereas with 2.0 mg/L KIN+1.5 mg/L NAA, it was 2.08 gm. After 6 weeks, it was 2.10 gm with KIN alone and 2.80 gm with KIN+NAA combination respectively. The result has been presented in Table 4.

Table 4. Comparative performance study of KIN and KIN+NAA in callus induction

Parameter under study	Hormonal performance		Hormonal conc.(mg/L)	
	KIN	KIN+NAA	KIN	KIN+NAA
Percentof explants showing callus induction	55.00	80.00	3.0	3.0+2.5
Days to callus induction	24.40	21.40	3.0	2.0+1.5
Weight of callus after 3 weeks (gm)	1.44	2.08	3.0	2.0+1.5
Weight of callus after 6 weeks (gm)	2.10	2.80	3.0	2.0+1.5

4.2 Sub-Experiment 2: Multiple shoot regeneration in Pineapple

For commercial cultivation, good quality planting material in large quantity is required. To fulfill the objectives of multiplication, the proliferated calli were cut into small pieces after 6 weeks of explants inoculation and subcultured into regeneration media. For shoot induction various phytohormones were used to find out the best treatment for commercial production. The results are presented separately under following headings –

4.2.1 Effect of BA on multiple shoot proliferation

The effect of different concentrations of BA have been presented under following headings with Figures 3-6 and Plate 6.

4.2.1.1 Percent of explants showed shoot induction

Significant variation was found on percent of explants showing shoot induction at different concentration of BA. The highest percentage (40 %) of shoot induction was induced in treatment 2.0 mg/L BA and the lowest percentage (25%) was induced in hormone free media (Figure 3). The treatment 1.0 mg/L & 3.0 mg/L BA also showed a good percentage for shoot induction and here is no significant variation was among them. Debargh (2006) obtained best multiplication with liquid MS medium, supplemented with 1.0 mg/L BA. Farahani (2014) found that higher multiplication rates for *Ananas comosus* L. were obtained with BA concentrations of 5.0 mg/L at 3 months.

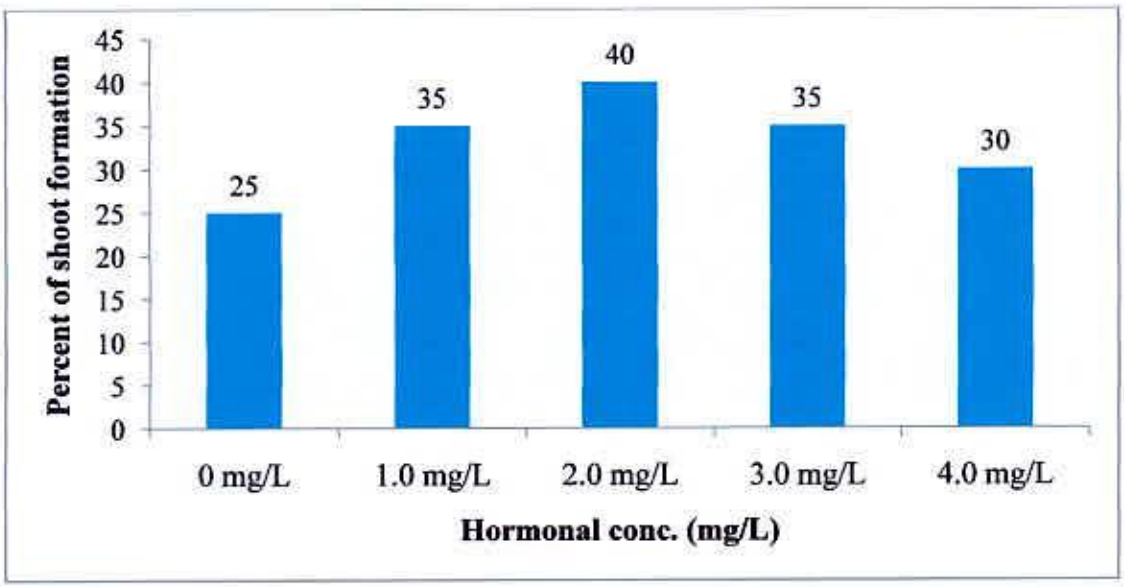


Fig.3. Percentage of explants showed shoot induction with BA

4.2.1.2 Days to shoot induction

With different concentrations of BA, significant variations were observed on days to shoot induction of pineapple. The maximum 24 days was recorded for shoot induction in control (0.0 mg/L). The second highest 16.40 days and 17.2 days were observed at 3.0 and 4.0 mg/L BA respectively. The minimum 15.6 days was required for shoot induction at 2.0 mg/L BA (Figure 4).

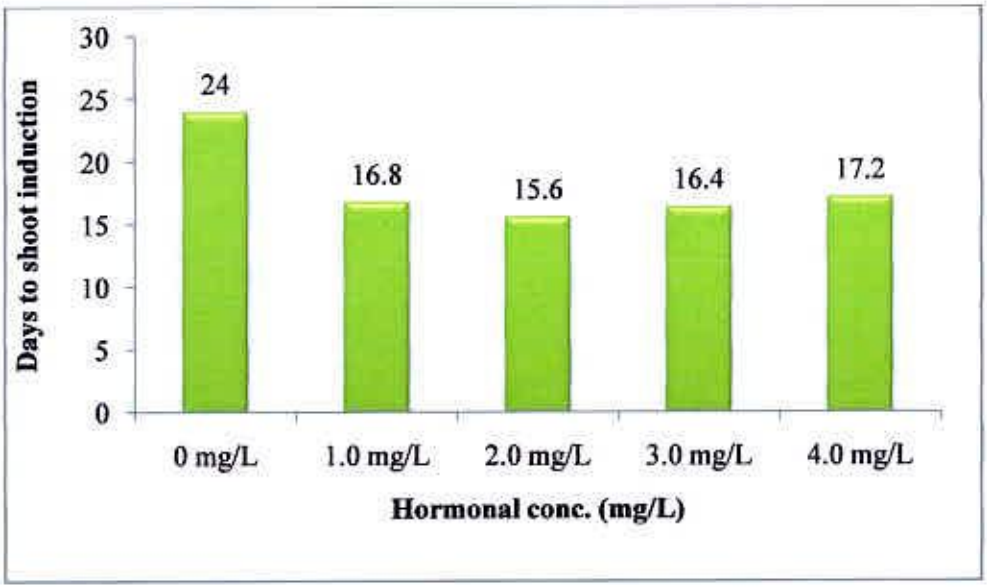


Fig.4. Effect of BA on days to shoot induction

4.2.1.3 Number of shoots per explant

Significant variations at 5% level were observed among different treatments of BA on number of shoots per explants of pineapple using MS media (Figure 5). After 3 and 6 weeks of subculture, the highest number of shoots (2.80 and 6.80 shoots per explants) were observed in treatment of 2.0 mg/L BA (Plate 6). The 3.0 mg/L of BA also showed good performance (2.20 and 4.80 no. shoots per explants) over the control. Ibrahim *et al.* (2013) showed highest 13.60 no. shoot/explants with 1.0 mg/L BA. Usman *et al* (2013) observed highest 11.5 no. shoot/explants at 5μM BA and any increase in BA concentration will result in the decline in proliferation of shoots until it reaches a point at which the effect become detrimental to the cell's ability to differenciate.

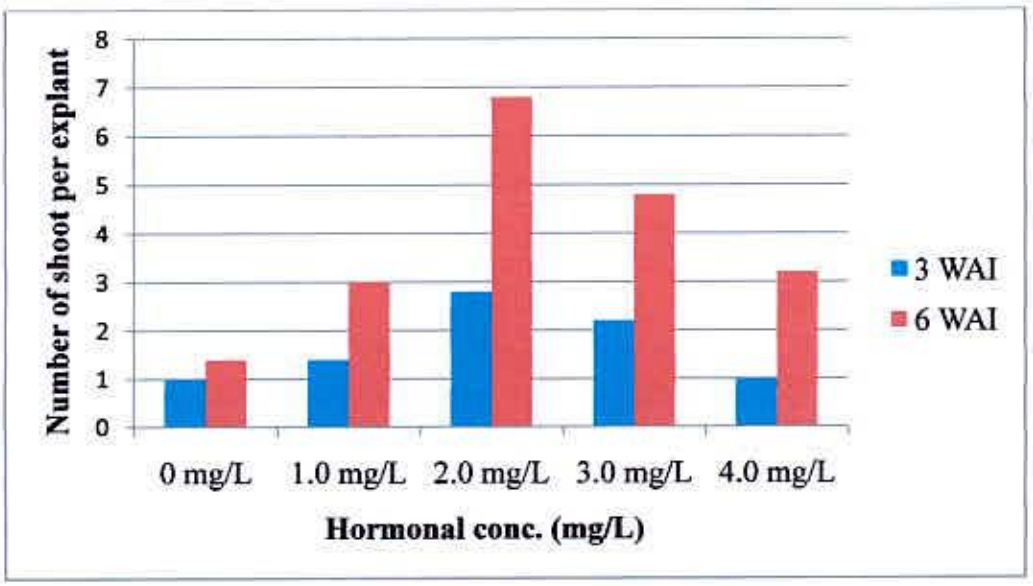


Fig. 5. Effect of BA on number of shoots per explant

4.2.1.4 Length of shoot (cm)

Result showed that the highest length of shoot (3.82cm and 5.86 cm) was observed in treatment of 2.0 mg/L BA after 3 and 6 weeks of subculture respectively. There is no significant significant variance on 2.0 mg/L and 3.0 mg/L BA on shoot length of pineapple after 3 weeks of subculture. The treatment 1.0 mg/L and 2.0 mg/L BA also showed same result after 6 weeks of subculture. On the other hand, the lowest length of shoot (2.3.12 cm) was observed in control after 3 and 6 weeks of subculture respectively which was statistically different from all other treatments. Usman *et al.* (2013) found 5.8 cm mean length shoot of pineapple at 5.0 µM after 2 months of inoculation.

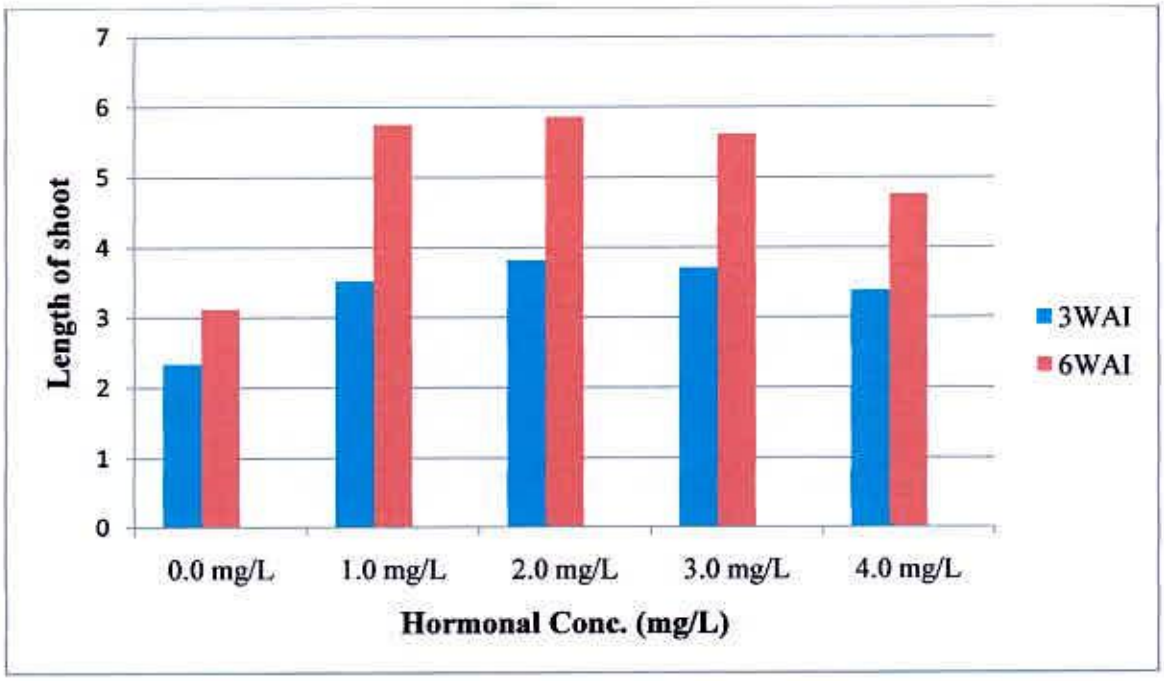
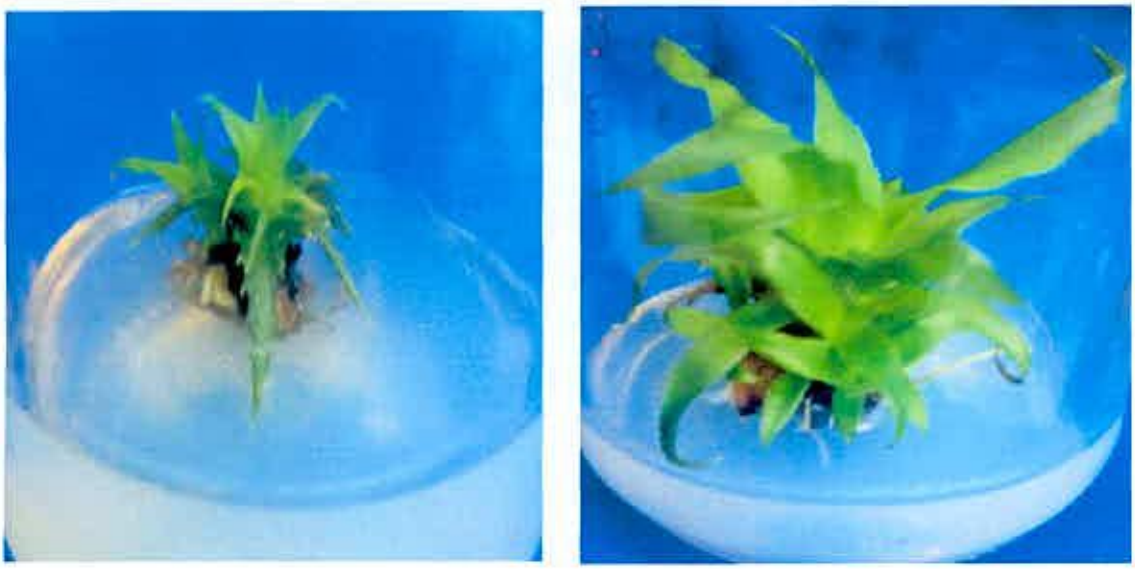


Fig.6. Effect of BA on length of shoot after 3 and 6 weeks of inoculation



A B

Plate 6. Shoot proliferation of pineapple on MS media supplemented with 2.0 mg/l BA (A) After 3 weeks and (B) 6 weeks of subculture.

4.2.2 The combine effect of BA + NAA on multiple shoot proliferation

The results of the combined effect of different concentrations of BA + NAA have been presented under following headings with Table 5-6 , Figure 7 and Plate 7.

4.2.2.1 Percent of explants showing shoot induction

There was significant influence of BA+ NAA concentrations on percent of explants showing shoot induction. The highest percentage (65%) of shoot induction was found in treatment 2.0 mg/L BA + 1.0 mg/L NAA and the lowest percentage (25%) was induced in hormone free media (Table 5). Akbar *et al.* (2006) found the highest 68% shoot induction with 1.5 mg/L BA and 0.5 mg/L NAA.

4.2.2.2 Days to shoot induction

Significant Variations were observed among different concentrations of BA + NAA on days to shoot induction. The maximum days to shoot induction was recorded in control (24 days) and 3.0 mg/L BA + 2.0 mg/L NAA required minimum 12.2 days (Table 5).

Table 5. Combined effect of BA+NAA on shoot induction potentiality

BA (mg/L)	NAA (mg/L)	No. of inoculated explants	No. explants Induced shoot	Percent of shoot formation	Days to shoot induction from callus
MS-Control		20	5	25	24 a
2.0	1.0	20	13	65	12.6de
	2.0	20	12	60	13.2cd
	3.0	20	11	55	13.8 bc
	4.0	20	9	45	14.2 b
3.0	1.0	20	10	50	14.2 b
	2.0	20	10	50	12.2 e
	3.0	20	9	45	11 f
	4.0	20	8	40	11.2 f
LSD (0.05)				0.99	0.8813
cv (%)				1.34	4.87

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

4.2.2.3 Number of shoots/explants

There was significant influence of different concentrations of BA + NAA on the number of shoots per explants. Data were recorded after 3 and 6 weeks of culture on MS media. The results have been presented in figure 7 and Plate 7. The treatment 2.0 mg/L BA + 1.0 mg/L NAA gave the highest number of shoots (5.6 and 10.6) after 3 and 6 weeks of subculture, respectively whereas the lowest number of shoots (1.40 and 2.34 after 3 and 6 weeks of subculture respectively) was found with hormone free media.

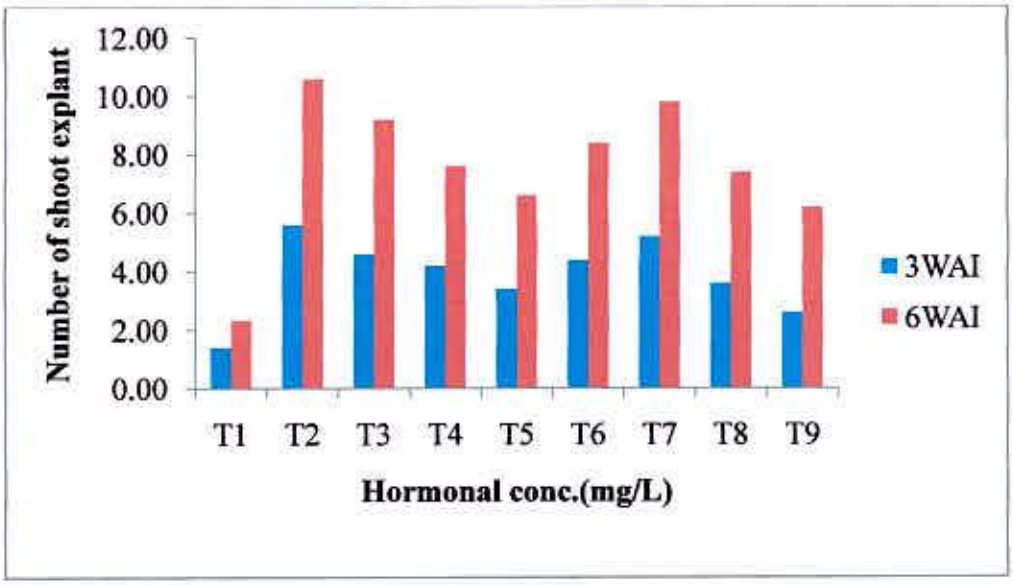


Fig.7. Effect of various combinations of BA and NAA on no. of shoots/ explants of pineapple after 3 and 6 weeks of inoculation

Hormonal conc. (mg/L):

T1=0+0	T3=2.0+2.0	T5=2.0+4.0	T7=3.0+2.0	T9=3.0+4.0
T2=2.0+1.0	T4=2.0+3.0	T6=3.0+1.0	T8=3.0+3.0	

4.2.2.4 Length of shoot (cm)

Result showed that the highest length of shoot 4.86cm was observed in treatment of 2.0 mg/L BA with 1.0 mg/L NAA after 3 weeks of subculture. There was no significant variance on 2.0 mg/L BA with 2.0 mg/L NAA on shoot length of pineapple after 3 weeks of subculture. But longest shoot (7.38 cm) was found in 3.0 mg/L BA with 3.0 mg/L NAA after 6 weeks of subculture. On the other hand, the lowest length of shoot (2.34cm and 3.12 cm) was observed in control (0.0 mg/L) after 3 and 6 weeks of subculture, respectively which was statistically different from all other treatments (Table 6). Amin *et al.* (2005) found 4.28 cm shoot length with 2.0 mg/L BA in combination with 1.0 mg/L NAA after 6 weeks of culture.

Table 6. Combined effect of BA and NAA on length of shoot of pineapple

BA (mg/L)	NAA (mg/L)	Length of shoot after 3 weeks (cm)	Length of shoot after 6 weeks(cm)
MS-Control		2.34e	3.12f
2.0	1.0	4.86a	6.38b
	2.0	4.77ab	6.16cd
	3.0	4.69bc	5.95de
	4.0	4.48d	5.92e
3.0	1.0	4.61c	6.30bc
	2.0	4.66c	6.34bc
	3.0	4.50d	7.38a
	4.0	4.45d	6.06de
LSD (0.05)		0..09	0.20
cv (%)		1.61	2.61

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

A B

Plate 7. Shoot proliferation of pineapple on MS media supplemented with 2.0 mg/L BA +1.0 mg/L NAA .A. After 3 Weeks and B. After 6 weeks of subculture

4.2.3 Effect of KIN on multiple shoot proliferation

For multiple shoot induction, MS supplemented with KIN alone or in combination with NAA or IBA were studied. Most of the researcher used it to develop an efficient protocol. Akbar *et al.*, 2006; Roy *et al.*, 2000 also reported that it is good phytohormone for pineapple micro-propagation. The results of the effect of different concentrations of KIN have been presented under following headings with Table 7-9 and Plate 8. .

4.2.3.1 Percent of explants showing shoot induction

It has been observed that 2 mg/L and 3 mg/L KIN shows highest percentage (45 % and 50%) of shoot induction respectively and the lowest percentage (25%) was induced in hormone free media (Table 7). Akbar *et al* (2003) reported 30% of explants showing shoot induction with MS supplemented with 1.5 mg/L KIN.

4.2.3.2 Days to shoot induction

For commercial cultivation, it is important to supply planting material within short time. That's why, it is needed to develop a protocol to propagate planting material with less time required. Kinetin hormone is probably best choice for it. Significant variations were observed among different concentrations of KIN on days to shoot induction. The maximum days to shoot induction were recorded in control (24 days) and 3.0 mg/L required minimum 12 days only after subculture of callus (Table7).

Table 7. Effect of KIN on shoot induction potentiality

Growth regulator	Concentration (mg/L)	Percent of shoot formation	Days to shoot induction from callus
Control	0.0	25	24.00a
KIN	1.0	30	14.60b
	2.0	45	12.80c
	3.0	50	12.00d
	4.0	40	14.40b
LSD (0.05)			0.75
CV (%)			3.61

4.2.3.3 Number of shoots per explants

There was significant influence of different concentrations of KIN on the number of shoots per explant. Data were recorded after 3 and 6 weeks of subculture on MS media with KIN. The results have been presented in Table 8. However, 3.0 mg/L KIN gave the highest number of shoots (4.80 and 9.60 at 3 weeks and 6 weeks of subculture of callus respectively) whereas the lowest number of shoots (1.4 and 2.34 after 3 weeks and 6 weeks of subculture of callus respectively) was found with hormone free media (Plate 8). Variations in shoot proliferation due to KIN conc. were also reported by Akbar *et al.* (2003) and Roy *et al.* (2010). Akbar *et.al* (2003) noticed 1.9 shoots per explants in media supplemented with 1.5 mg/L KIN after two months of inoculation. Ibrahim *et al* (2013) found 8.60 no. of shoot/explants with 1mg/L KIN after 2 months of inoculation.

Table 8. Effect of KIN on no. of shoot/explants

Growth regulator	Concentration (mg/L)	Number of shoot per explants	
		3 WAI	6 WAI
control	0.0	1.40e	2.34e
KIN	1.0	1.60d	4.40d
	2.0	4.20b	7.40b
	3.0	4.80a	9.60a
	4.0	3.20c	6.20c
LSD (0.05)		0.50	0.64
CV (%)		12.64	8.18

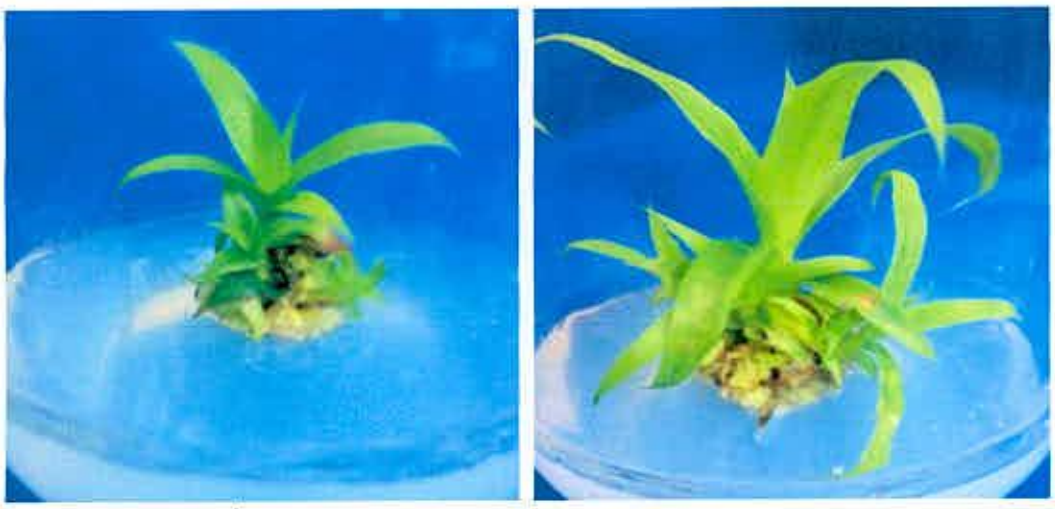
Values in the column are the means of five replicates. In a column, mean values with the same letters are not statistically different from each other at 5% probability by DMRT

4.2.3.4 Length of shoot (cm)

Different treatments of KIN showed significant variations on length of shoot (cm) of pineapple at 5% level of significance (Table 9). The highest length of shoot (4.24cm and 6.96 cm) was observed in treatment of 3.0 mg/L KIN after 3 and 6 weeks of subculture respectively which is statistically different from the other treatment. On the other hand, the lowest length of shoot (2.34 and 3.14 cm) was observed in control (0.0 mg/L) KIN after 3 and 6 weeks of subculture, respectively.

Table 9. Effect of KIN on length of shoot of pineapple

Growth regulator	Concentration (mg/L)	Length of shoot After 3 weeks (cm)	Length of shoot After 6 weeks (cm)
control	0.0	2.34e	3.12d
KIN	1.0	3.77d	6.60b
	2.0	3.96c	6.74b
	3.0	4.24a	6.96a
	4.0	4.11b	6.30c
LSD (0.05)		0.13	0.17
CV (%)		2.68	2.19



A B

Plate 8. Shoot proliferation of pineapple on MS media supplemented with 3.0 mg/ L KIN (A) After 3 weeks (B) After 6 weeks of subculture

4.2.4. The combine effect of KIN+NAA on multiple shoot proliferation

The combine effect of KIN+NAA on shoot proliferation and elongation of pineapple was investigated by adding different concentrations of KIN and NAA to a basal MS medium (semi-solid).For shoot induction, 1.0 gm of of callus was cultured on the KIN+NAA containing medium. The result of the combined effect of different concentrations of KIN+NAA have been presented under following headings with Tables10-11, Figure 8 and Plate 9.

4.2.4.1 Percent of explants showing shoot induction

There was significant variation of KIN+NAA concentration on percent of explants showing shoot induction. The highest percentage (85%) of shoot induction was induced in treatment 3.0 mg/L KIN + 1.0 mg/L NAA and the lowest percentage (25%) was induced in hormone free media (Table 10). 3.0 mg/L KIN+ 1.5 mg/L NAA combination also shows good percentage of shoot induction. Akbar *et.al* (2003) found 78% shoot induction potentiality at 1.5 mg/L KIN+ 1 mg/L NAA combination. M.Fitchet-Prunell (1993) also developed a protocol with 2.0 mg/L KIN+2.0 mg/l NAA combination showing good shoot induction potentiality.

4.2.4.2 Days to shoot induction

With different concentrations of KIN+ NAA, significant variations were observed on days to shoot induction of pineapple. The highest 24 days was recorded for shoot induction in control (0.0 mg/L). The second highest days (11.60) and 11.20 was observed at 3.0 and 4.0 mg/L NAA with 3.0 mg/L KIN respectively and the minimum 7.60 days was required for shoot induction at 3.0 mg/L KIN with 1.0 mg/L NAA. Moreover, it can be concluded that high conc. of NAA is not effective in combination with KIN.

Table 10. Combined effect of KIN+NAA on shoot induction potentiality

KIN +NAA (mg/L)	Percent of shoot formation	Days to shoot induction from callus
control	25	24.00a
2.0+1.0	55	8.60def
2.0+1.5	60	8.20ef
2.0+2.0	50	9.20cde
3.0+1.0	85	7.60f
3.0+1.5	80	9.60cd
3.0+2.0	70	10.20c
3.0+3.0	65	11.20b
3.0+4.0	55	11.60b
LSD (0.05)		0.99
cv (%)		6.93

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

4.2.4.3 Number of shoots per explant

Significant variations at 5% level were observed among different concentration of KIN+NAA combination on number of shoots/explants of pineapple on MS media. After 3 and 6 weeks of subculture, the highest number of shoots observed were 10.80 and 15.60 no. shoots/explant, respectively, in treatment of 3.0 mg/L KIN with 1.0 mg/L NAA. There was no significant variation 3.0 mg/L KIN with 1.5 mg/L NAA and the second highest (10.20 and 14.40 shoots/explants) was observed from it after 3 and 6 weeks of subculture respectively (Fig.8 & Plate 9). Akbar *et al.* (2003) observed 5.7 no. shoots/explants after two months of inoculation at 1.5 mg/L KIN with 0.5 mg/L NAA.

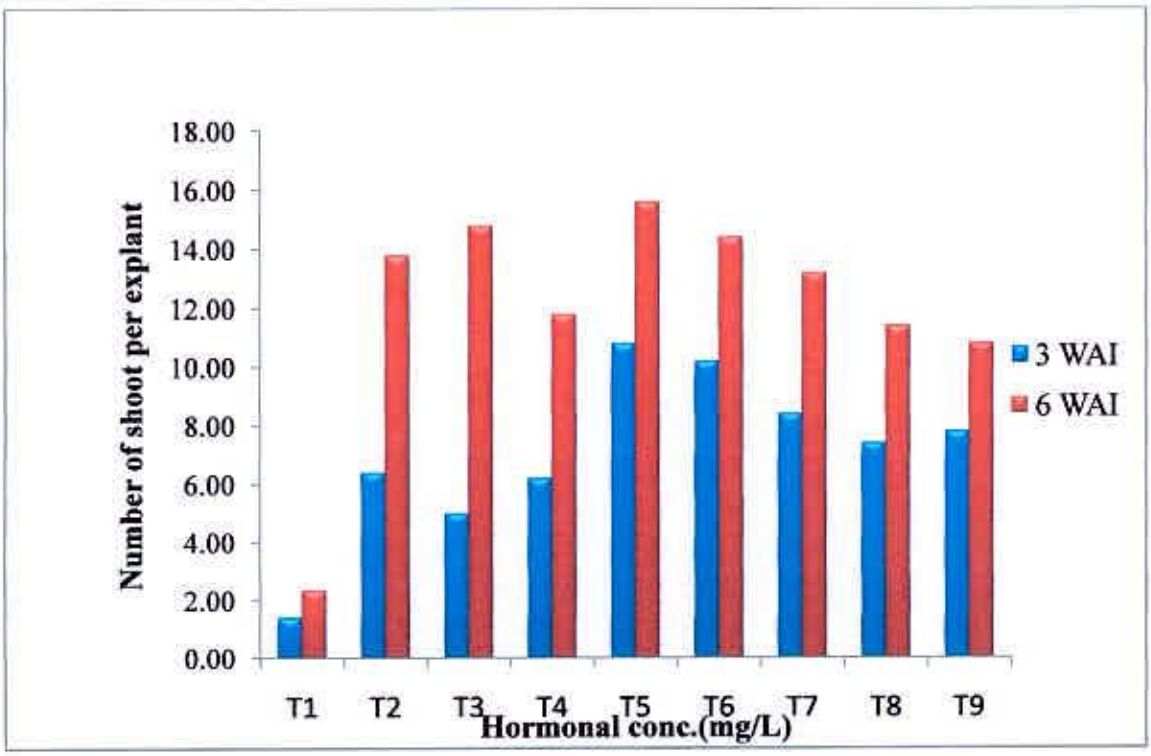


Fig.8. Combined effect of KIN+NAA on no .of shoots/explant

Hormonal conc. (mg/L):

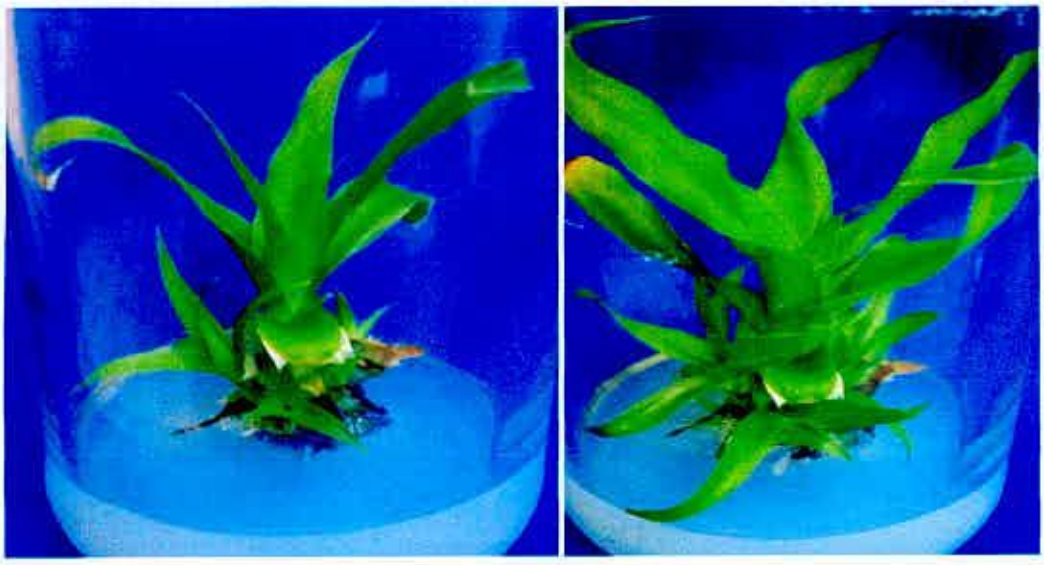
T1=0+0	T4=2.0+2.0	T7=3.0+2.0
T2=2.0+1.0	T5=3.0+1.0	T8=3.0+3.0
T3=2.0+1.5	T6=3.0+1.5	T9=3.0+4.0

4.2.4.4 Length of shoot (cm)

Different treatments of KIN +NAA showed significant variations on length of shoot (cm) of Pineapple at 5% level of significance (Table 11). The highest length of shoot (5.18 and 7.92 cm) was observed in treatment of 3.0 mg/L KIN with 1.0 mg/L NAA after 3 and 6 weeks of subculture, respectively. A combination of 3.0 mg/L KIN with 1.5 mg/L NAA combination showed second highest shoot length (4.94 cm and 7.72 cm) respectively. On the other hand, the lowest length of shoot (2.34 cm and 3.12 cm) was observed in control (0.0 mg/L), 3 and 6 weeks of subculture, respectively which was statistically different from all other treatments.

Table 11. Combined effect of KIN+NAA on length of shoot of pineapple

KIN+NAA (mg/L)	Length of shoot after 3 weeks (cm)	Length of shoot after 6 weeks (cm)
MS-control	2.34f	3.12h
2.0+1.0	4.59de	7.14d
2.0+1.5	4.68d	7.78b
2.0+2.0	4.48e	7.50c
3.0+1.0	5.18a	7.92a
3.0+1.5	4.94b	7.72b
3.0+2.0	4.82c	6.94e
3.0+3.0	4.68d	6.64f
3.0+4.0	4.52e	6.46g
LSD (0.05)	0.11	0.08
cv (%)	1.85	2.97



A B

Plate 9. Shoot proliferation of pineapple on MS media supplemented with 3.0 mg/ L KIN +1.0 mg/L NAA A. After 3 weeks B. After 6 weeks of subculture

4.2.5 The combined effect of KIN + IBA on multiple shoot proliferation

The results of the combined effect of different concentrations of BAP and IBA have been presented under following headings with Tables 12-13, Figures 9-10 and Plate 10.

4.2.3.1 Percent of explants showing shoot induction

There was significant variation of KIN +IBA combination on percent of explants showing shoot induction. The highest percentage (80%) of shoot induction was induced in treatment 3mg/l KIN with 2 mg/l IBA. Minimum percentage (25%) was induced in hormone free media (Table 12).

Table 12. Combined effect of KIN+IBA on shoot induction potentiality of pineapple

KIN+ IBA (mg/L)	No. of explants inoculated	Number of explants initiated shoot	Percent of shoot formation
MS-control	20	5	25
2.0+1.0	20	12	60
2.0+2.0	20	14	70
2.0+3.0	20	15	75
2.0+4.0	20	13	65
3.0+1.0	20	15	75
3.0+2.0	20	16	80
3.0+3.0	20	14	70
3.0+4.0	20	12	60

4.2.5.2 Days to shoot induction

Variations were observed among different concentration of KIN+IBA on days to shoot induction. The maximum days to shoot induction were recorded in control (24 days) and 3.0 mg/L KIN + 2.0 mg/L IBA required minimum 8.80 days.

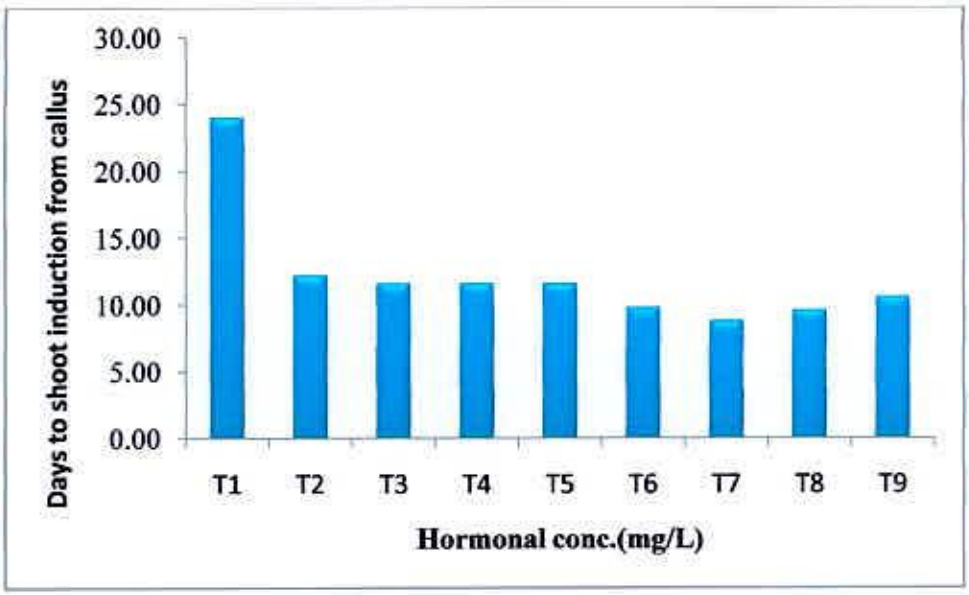


Fig. 9. Combined effect of KIN+IBA on days to shoot induction from callus

Hormonal conc. (mg/L):

T1=0+0	T4=2.0+3.0	T7=3.0+2.0
T2=2.0+1.0	T5=2.0+4.0	T8=3.0+3.0
T3=2.0+2.0	T6=3.0+1.0	T9=3.0+4.0

4.2.5.3 Number of shoots per explants

There was significant influence of different concentrations of KIN + IBA on the number of shoots per explant. Data were recorded after 3 and 6 weeks of subculture of callus on MS media with KIN + IBA combination. The results have been presented in Figure10.The combination of 3 mg/L KIN + 1.0 mg/L IBA gave the highest number of shoots (12.40 and 18.60 after 3 and 6 weeks of subculture respectively) which was statistically different from all other hormonal concentrations whereas the lowest number of shoots (1.0 and 1.40 after 3 and 6 weeks of subculture respectively) was found with hormone free media (Fig.10 & Plate10).

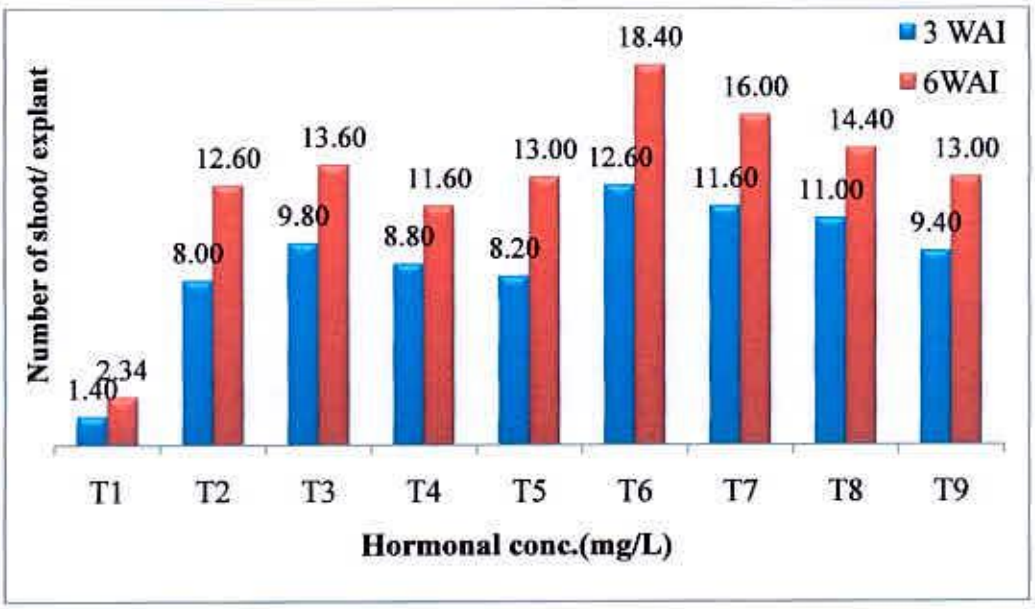
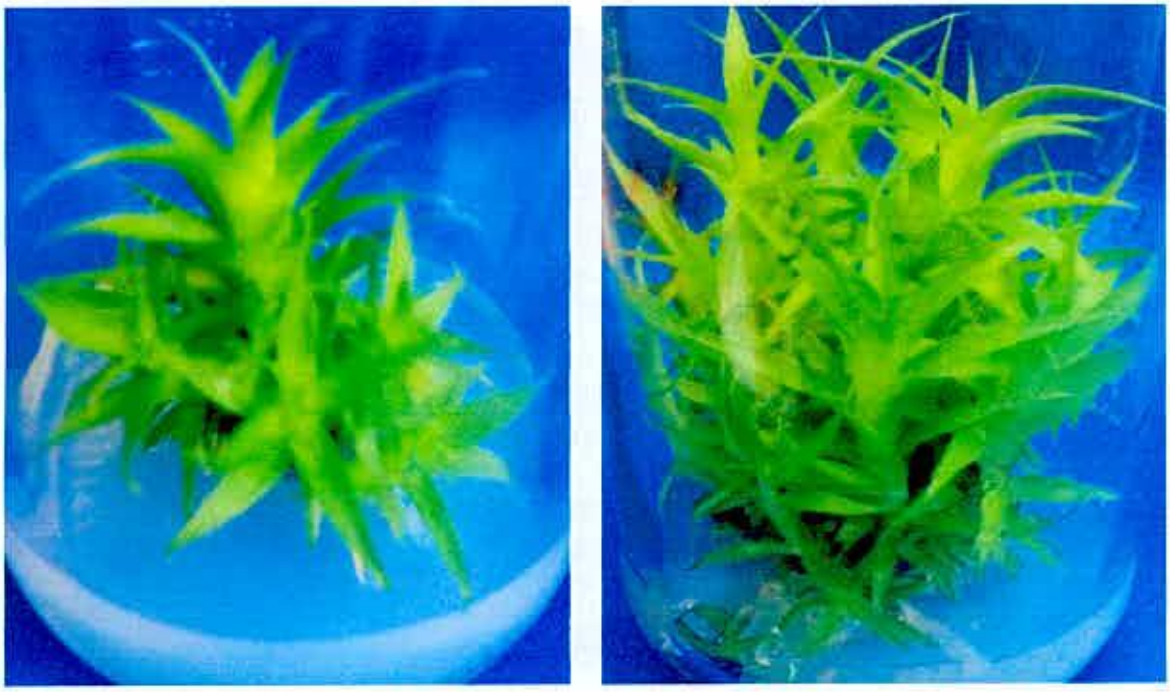


Fig. 10. Combined effect of KIN+ IBA on no. of shoot per explants of pineapple after 3 and 6 weeks of subculture

Hormonal conc. (mg/L):

T1=NIL	T4=2.0+3.0	T7=3.0+2.0
T2=2.0+1.0	T5=2.0+4.0	T8=3.0+3.0
T3=2.0+2.0	T6=3.0+1.0	T9=3.0+4.0



A B

Plate 10. Shoot proliferation of pineapple on MS supplemented with 3.0 mg/L KIN +1.0 mg/L IBA (A). After 3 and (B) 6 weeks of subculture of callus

4.2.5.4 Length of shoot (cm)

Different treatments of KIN+IBA showed significant variations on length of shoot (cm) of Pineapple at 5% level of significance (Table 13). The highest length of shoot (6.76cm and 9.04 cm) was observed in treatment of 3.0 mg/L KIN with 2 mg/L IBA after 3 and 6 weeks of subculture respectively (Plate 11). On the other hand, the lowest length of shoot (2.34cm and 3.12 cm) was observed in control (0.0 mg/L) after 3 and 6 weeks of subculture respectively which was statistically different from all other treatments.

Table 13. Combined effect of KIN+IBA on length of shoot of pineapple

KIN +IBA (mg/L)	Length of shoot after 3 weeks (cm)	Length of shoot after 6 weeks (cm)
Ms-control	2.34h	3.12h
2.0+1.0	4.74f	7.41g
2.0+2.0	5.10e	7.90d
2.0+3.0	4.58g	7.46g
2.0+4.0	4.58g	7.46g
3.0+1.0	5.74 d	7.82e
3.0+2.0	6.76a	9.04a
3.0+3.0	6.62b	8.54b
3.0+4.0	6.44c	8.16c
LSD (0.05)	0.13	0.06
cv (%)	1.90	2.59

4.2.6 The comparative performance of different growth hormone on shoot proliferation

The comparative performance of various growth hormones revealed variation in respect of all parameters under studied. The result has been presented in Tables 14-15, Figure11-12 and Plate 11.

4.2.6.1 Percent of explants showing shoot induction

Significance variation was observed among the different type of hormone on percent of explants showed shoot induction. The highest (85%) shoot induction was achieved with the 3.0 mg/L KIN + 1.0 mg/L NAA and the lowest (40%) was in case of 2.0 mg/L BA (Fig.11).The treatment 3.0 mg/L KIN + 2.0 mg/L IBA also showed good percentage (80.0%) of shoot induction. Moreover, it is observed that KIN with combination of NAA and IBA perform better than KIN alone.

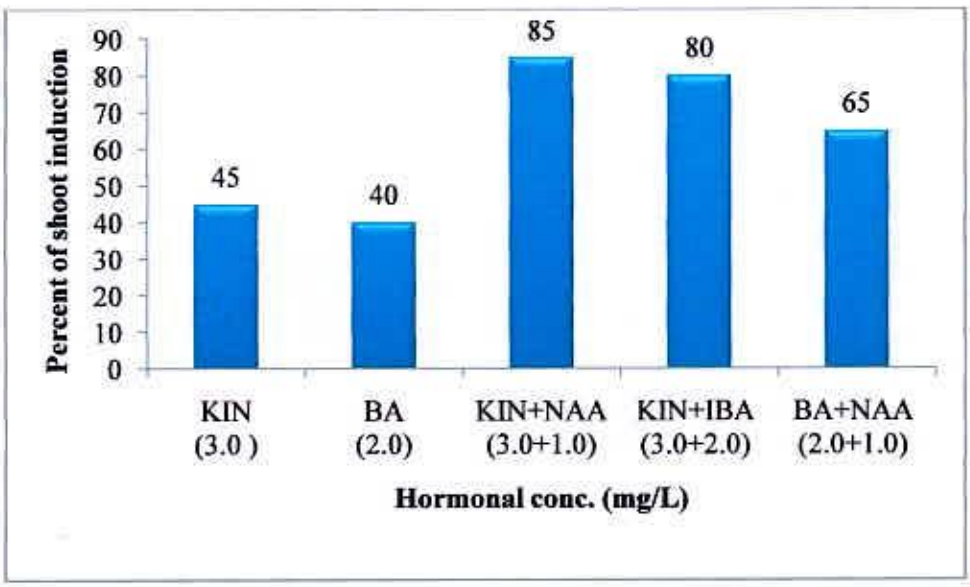


Fig.11.The comparative performance of growth hormone on percent of explants showing shoot induction

Akbar *et al.* (2003) reported that BA +NAA combination was not so efficient to initiate shoot regeneration from callus as compared to KIN-NAA combination. The percentage of calli forming shoots, and the number of regenerated shoots per callus varied according to the growth regulator treatment. MS containing KIN alone resulted in shoot differentiation, more efficiently than that observed in BA, but at a lower frequency compared to NAA supplemented media.

4.2.6.2 Days to shoot induction

To fulfill the objective of supplying planting material within short time, it is needed to find out the hormonal dose of minimum days required. The results of major effect of different types and combinations of hormone on shoot induction potentiality have been presented Table 14. Significance variations were observed among these hormones. Maximum15.6 days to shoot induction was recorded in 2.0 mg/L BA whereas the lowest 7.60 days was required in media containing 3.0 mg/L KIN + 1.0 mg/L NAA.

Table 14. Comparative performance of growth hormone of pineapple on days to shoot induction

Name of the Phytohormones	Conc. (mg/L)	Days to shoot Induction from callus
BA	2.00	15.60
KIN	2.00	12.80
BA + NAA	3.0 + 3.0	11.00
KIN + NAA	3.0 + 1.0	7.60
KIN+IBA	3.00+2.00	8.80

4.2.6.3 Number of shoot per explants

The results of comparative study of major hormonal doses on number of shoot per explant have been presented in Fig.12. Cytokinin level produced a significant response upon the number of shoot per explant and also showed influence on production of the numbers shoots/explant. The highest number of shoot 18.60 was found with 3.0 mg/L KIN + 1.0 mg/L IBA and the lowest 6.8 with 2.0 mg/L BA after 6 weeks of subculture.

Ibrahim *et al.* (2013) showed that MS medium supplemented with kinetin at 0.5 or 1.0 mg/L was superior to BA in all vegetative characteristics of shoot formation except the treatment of kinetin at 1.0 mg/L which was not significantly different from 1.0 mg/L BA. It may be due to more effectiveness of KIN than BA in stimulation of adventitious shoots production.

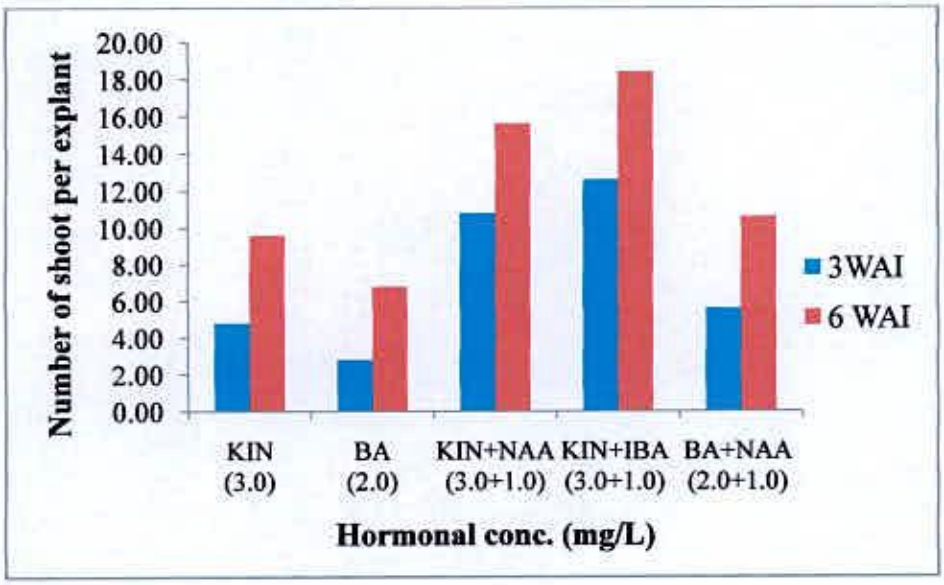


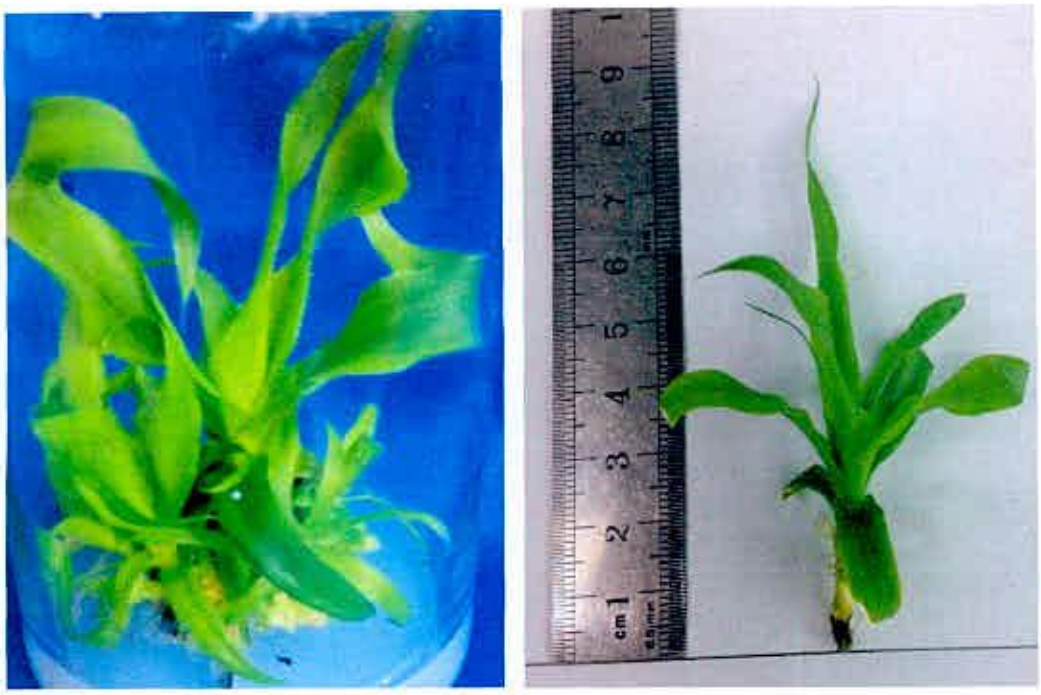
Fig.12. Comparative performance of different growth hormone on no. of shoot/explants after 3 & 6 weeks of inoculation

4.2.6.4 Length of shoot (cm)

There was significant influence of different hormone combinations and concentrations on length of shoot (cm). The comparative results of different growth hormones have been presented in Table 15 and Plate 11. The highest (6.76 cm and 9.04 cm)) average length of shoot was obtained with 3.0 mg/L KIN+2.0 mg/L IBA whereas the lowest (3.82 cm and 5.86 cm) length of shoot in 2.0 mg/L BA.

Table 15. The comparative performance of growth hormone on average length of shoot (cm)

Name of the Phytohormones	Phytohormones concentration (mg/L)	Length of shoot (cm) after 3 weeks	Length of shoot (cm) after 6weeks
BA	2.0	3.82	5.86
KIN	3.0	4.24	6.96
BA + NAA	3.0+3.0	4.85	7.38
KIN + NAA	3.0+1.0	5.18	7.92
KIN+IBA	3.0+2.0	6.76	9.04

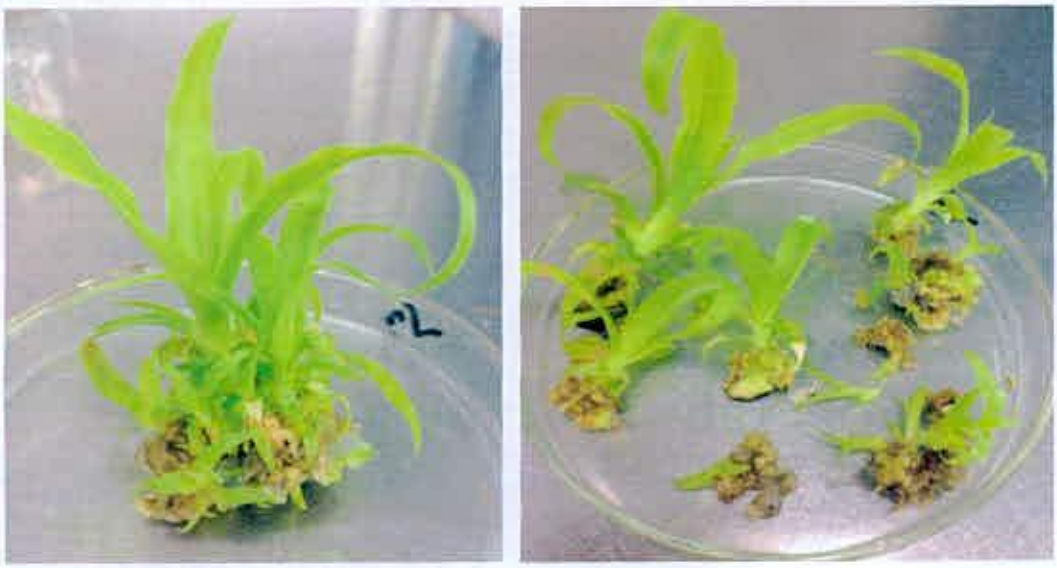


A B

Plate 11. Longest shoot of pineapple on MS media supplemented with 3.0 mg/L KIN + 2.0 mg /L IBA A. Longest shoot in culture medium B. 9.04 cm long shoot

4.3 Sub-Experiment 3: Root induction in Pineapple

The rooting hormone IBA, NAA and combination of IBA+NAA were applied. Root formation was not observed when shoots were cultured on a medium lacking auxin. In contrast root formation was observed when regenerated shoots were cultured on half strength MS supplemented with IBA or NAA alone, in combination IBA+NAA. Bhatia and Ashwath (2002) observed that rooting could occur in the multiplication medium if the stages continued for more than 6 subcultures. These reports confirmed our results that shoot age is an important factor for *in vitro* rooting of pineapples. The rooting response varied with type and concentration of auxin. The results of experiment have been presented under different heading utilizing Fig. 13-16, Tables 16-18 and Plates 12-13.



A B

Plate 12. Transfer of regenerated shoot to root induction media
A. Plantlets in petridish B. Separation of plantlets by sterilized blade

4.3.1 Root formation of pineapple with IBA or NAA alone

Generally, the different cultivars and medium strength play major role in rooting of pineapple. In a solid MS medium, 0.5 mg/L IBA was recommended by Dolgov *et al.*, 1998; Fernando, 1986. IBA at 1.0 mg/L was recommended for Madhupur cultivar (Akbar *et al.*, 2003) and Queen Pineapple (Devi *et al.*, 1997).

4.3.1.1 Percent of explants showing root induction

There was considerable variation among growth regulators on percentage of explants showing root induction. The results have been shown in Fig.13. The highest percentage (60%) of root induction was recorded with 2.0 mg/L IBA, whereas 55% root induction was found in 2.0 mg/L NAA. The lowest percentage (20%) of root induction was recorded in media lack of plant growth regulator. Furthermore, it is noticed that IBA performed a little better than NAA in respect of percentage of root formation. Hamid *et al.* (2013) reported that full strength solid medium enriched with 1.0 mg/L NAA was the best choice for rooting of both Smooth Cayenne and Morris variety of pineapple.

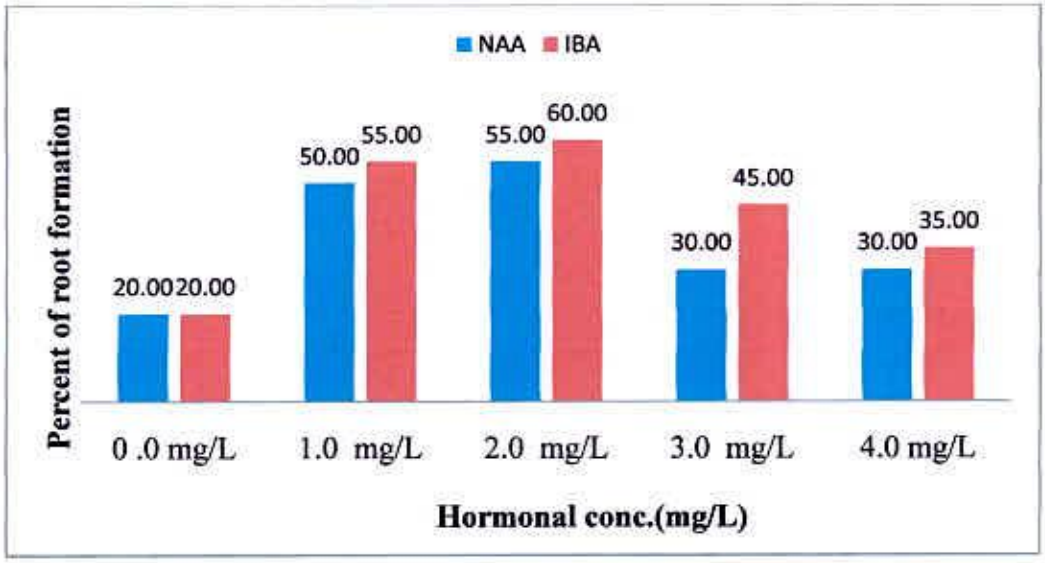


Fig. 13. Effects of IBA and NAA on percent of explants showed root induction

However, the higher concentration above 2.0 mg/L exerted its inhibitory effect on the rooting percentage in media with NAA. The inhibiting effect might be due to higher concentration induces the higher level of degradative metabolites in tissue, which may block the regeneration process (Baker and Wetstein, 2004). Numerous studies also supported the usefulness of IBA as the most effective auxin 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 NAA, IAA or synthetic auxins.

4.3.1.2 Days to root induction

Hormonal concentration has significant level of variation on days for root induction. The highest 34.40 days to root induction was required in media lack of growth regulator. The lowest 21.80 days in case of 2.0 mg/L IBA and 25.20 days was required by 2 .0 mg/L NAA (Table 16).

4.3.1.3 Number of roots per explants

The significant variations were observed among different treatments of IBA and NAA on growth parameters of pineapple such as number of root after 3 and 6 weeks of subculture (Table 16). The highest number of root (5.40 and 11.80 per explants) after 3 and 6 weeks of subculture respectively was produced by 2.0 mg/L of IBA. On the other hand, the lowest number of root (0.40 and 0.80 per explants) was produced by the untreated control.

Table 16. Effect of IBA root induction potentiality of pineapple

Name of hormone	Concentration (mg/L)	Days to root induction	Number of roots/explant	
			3WAI	6WAI
Control	0	34.40a	0.40e	0.80e
IBA	1.0	27.60b	3.20b	3.20d
	2.0	21.60e	5.40a	11.80a
	3.0	24.00d	2.80c	7.80b
	4.0	26.00c	2.10d	3.80c
NAA	1.0	22.60c	5.20a	9.20a
	2.0	25.20b	3.80b	6.20b
	3.0	26.80b	1.60c	4.20c
	4.0	26.80b	1.40c	3.80c

In case of NAA, the highest number of root (5.20 and 9.20 per explant) was produced by 1.0 mg/L of NAA after 3 and 6 weeks of subculture. On the other hand, the lowest number of root was produced by the untreated control.

According to Khan *et al*.(2004) where several concentrations of IBA were employed in the greatest average number of roots were 5.00 in a media containing 1.0 mg/L IBA. However, when IBA was substituted by NAA, it was observed that at 1.0 mg/L NAA, although the percentage of rooting was 86%, the average number of roots was 3.55 which also required the shortest time of 8-15 days for root induction. Result supported using1.0 mg/L NAA in full strength medium for pineapple as recommended by Hamad and Taha (2008) and Fitchet (1993) at 2.0 mg/L IBA.

4.3.1.4 Length of root (cm)

Both IBA and NAA play a significant role on average length of root per explant (cm). The highest root length (4.94 cm) was obtained from 2.0 mg/L IBA (Fig. 14) and 4.84 cm in case of 1.0 mg/L NAA after 6 weeks of subculture. No significant variation was observed between 2 .0 mg/L and 3.0 mg/L IBA. The minimum 1.90 cm length of root (cm) was in control.

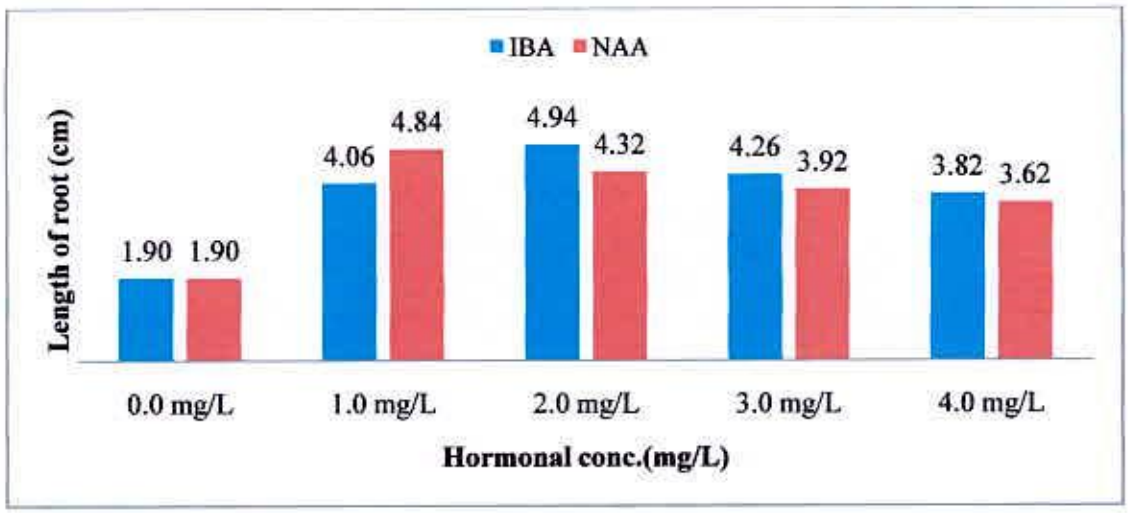


Fig.14. Effect of IBA and NAA on length of root of pineapple

The tallest plantlets of Smooth Cayenne (99.2 mm) was obtained in full strength medium enriched with 1.0 mg/L NAA, while the tallest of Morris (58.3 mm) was obtained in quarter strength enriched with 2.0 mg/L IBA by Hamid *et al.*(2013).

The researchers studied the *in vitro* induction and proliferation of adventitious roots in pineapple by using different concentrations of IBA and NAA. They described that the MS solid medium enriched with 1.0 mg/L NAA was the best choice for rooting resulting in the tallest plantlets, highest number of roots per shoot and intermediate rooting percentage. However, Akbar *et al.* (2003) reported that IBA was found to be the best treatment for root induction. This might be caused by the cultivar of pineapple.

4.3.2 Combined effect of IBA and NAA on root growth and development

Root induction by the combined treatment of NAA+IBA showed significant variation for root growth and development. Result showed that minimum 16 days were required to root initiation with 2.0 mg/L IBA + 1.5 mg/L NAA. No statistical variation was found with 2.0 mg/L IBA + 1.0 mg/L NAA also for root initiation. On the other hand, maximum 34.40 days were required in hormone free media. Moreover, maximum 75.00% root induction was observed with same hormonal treatment. Minimum 20 % root induction was found in control. This result is different from Akbar *et al.* (2003). He found maximum 59.3% root induction and minimum days for root initiation with 2.0 mg/L IBA+ 1.5 mg/L NAA. It is observed that no. of root/explants (9.60 and 15.60) is high with 3 mg/L IBA +1.0 mg/L NAA after 3 and 6 weeks of subculture respectively. No. of root is less in hormone free media. Longest root (7.0 cm) was found with 2.0 mg/L IBA + 1.5 mg/L NAA (Table 17 and Plate 13). So, it can be said that 2.0 mg/L IBA + 1.5 mg/L NAA combination is better than any other combination of hormone considering all growth parameter.

Half strength solidified MS media enriched with combination of 0.5 mg/L IBA and 0.5 mg/L NAA showed suitable result of rooting process (Firoozabady and Gutterson, 2003) .

Table 17. Combined effect of IBA+NAA on root growth and development

Combination of growth regulator	Concentration (mg/L)	Days to root induction	Percent of root formation	Number of root per explant 4 weeks	Number of root per explant 5 weeks	Length of root
Control	0+0	34.40 a	20	0.40 e	0.80 e	0.00 g
IBA+NAA	2.0+1.0	17.40 e	70	3.40 d	5.80 d	6.46 b
IBA+NAA	2.0+1.5	16.00 e	75	4.80 c	8.20 c	7.00 a
IBA+NAA	2.0+2.0	19.20 d	65	3.00 d	6.20 d	6.26 c
IBA+NAA	3.0+1.0	21.00 c	60	9.60 a	15.60 a	5.96 d
IBA+NAA	3.0+1.5	21.20 c	55	7.80 b	13.60 b	5.62 e
IBA+NAA	3.0+2.0	24.60 b	50	4.60 c	7.00 d	5.38 f
LSD (0.05)		1.47		0.82	1.16	0.06
cv (%)		5.11		13.02	10.86	3.77

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

4.3.3 Comparative performance of different rooting hormone

It has been observed that combined effect of 2 mg/L IBA +1.5 mg/L NAA performed better than NAA or IBA alone. It required less days (16 days) for root initiation, produce highest percentage of root (75.0), longest root (7.0 cm) from other treatment. Highest no. of root (9.60 and 15.60) was found with 3.0mg/L IBA+ 1.0 mg/L NAA after 3 and 6 weeks of subculture.

Table 18. Comparative performance of different rooting hormone

Combination of growth regulator	Conc mg/L	Days to root induction	Percent of root formation	Number of root/explant after 3weeks	Number of root/explants after 6 weeks	Length of root (cm)
Control	0.0	34.40	20.00	0.40	0.80	1.90
NAA	1.0	22.60	50.00	5.20	9.20	4.84
IBA	2.0	21.80	60.00	5.40	11.80	4.54
IBA+NAA	2+1.5	16.0	75.00	4.80	8.20	7.0
IBA+NAA	3+1	21.0	60.00	9.60	15.60	5.96

A B

Plate 13. Best root development with IBA+ NAA combination. (A) Highest No. of root on MS media with 3.0 mg/L IBA + 1.0 mg/L NAA (B) Longest root on MS media with 2.0 mg/L IBA + 1.5 mg/L NAA after 6 weeks of subculture

4.4 Experiment 4: Acclimatization of regenerated plantlets of pineapple

Plate 14. Regenerated plantlets prior to potting in growth chamber

4.4.1 Acclimatization in the culture room

The response of pineapple plantlets to hardening was also studied. After 45 days of culture on rooting media, the plantlets were taken for acclimatization. The small plantlets were taken out from culture vessel carefully without damaging any roots. Excess media around the root was washed off by running tape water to prevent further microbial infection. The plantlets were then transplanted in plastic pots. The pots were prepared from the mixture of Soil : Sand : Cow-dung (1:1:1) considering the physical characteristics, aeration and water holding capacity. To reduce sudden shock, the pot was kept in growth room for 7 days under controlled environment with 15 hour photoperiod and high relative humidity. Immediately after transplantation the plantlets were irrigated with a fine spray of water. The results of acclimatization showed that the 85% of plantlets survived in growth chamber (Table 19).

4.4.2 Acclimatization in the shade house

After 7 days of acclimatization in culture room, the plantlets were transferred into the shade house. Occasional spray of water was done to prevent sudden desiccations and maintain 75% humidity around the plantlets. Here survival rate was 82%.

4.4.3 Acclimatization in the open field

After 15 days the plantlets were kept in open environment, the pots were kept in direct sunlight and air for natural growth and development. After 30 days when the plants grew well and leaves turned reddish-green color, then they were planted in the field with soil pH 5.5 for fruit production. After proper hardening, survival rate was 78% in field condition (Table 19 and Plate 15. Usman *et al.* (2006) found that non-acid washed river side sand significantly produced more fully recovered plantlets (87.4%) at 4 wks after transplanting (4 WAT).

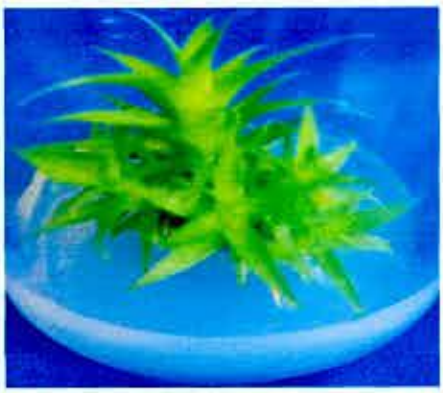
Table 19. Survival rate of *in vitro* regenerated plants of pineapple

Acclimatization	No. of plants transplanted	Duration of observation	No. of plants survived	Survival rate (%)
In growth chamber	20	7days	17	85
In shade house	17	15 days	14	82
In field condition	14	30 days	11	78

A B

Plate 16. Hardening of pineapple plant. A. In culture room

B. In open environment after 30 days



Chapter V
Summary and Conclusion

CHAPTER V

SUMMERY AND CONCLUSION

The present experiment was conducted in Biotechnology Laboratory of the Department of Biotechnology, Sher-e-Bangla Agricultural University, Sher-e-Bangla Nagar, Dhaka-1207 during the period from January, 2014 to December, 2014 to evaluate the effect of different plant growth regulators on *in vitro* callus induction, shoot regeneration and root formation along with acclimatization for *in vivo* survival of pineapple. The experiment was conducted at Completely Randomized Design (CRD) with 5 replications. KIN, BA were used as cytokinin and NAA, IBA was used as auxin group of hormone. The combined effect of hormones was also studied.

Fresh, healthy and disease free crown tip of 1.5-2 cm size was used as explants. Explants were sterilized with 70% ethanol for 1 minute, 10% NaOCl for 10 minutes and 5% NaOCl for 5 minutes with 1-2 drops Tween- 20.

It revealed that days to callus induction, size and weight of callus were significantly influenced with KIN alone or in combination with NAA. The highest percentage (80%) of callus induction was observed with 3.0 mg/L KIN+2.5 mg/L NAA treatment whereas the lowest (40%) for 1.0 mg/L KIN alone. Minimum 21.20 days were required to callus initiation in 2.0mg/L KIN + 1.5 mg/L NAA treatment and maximum 27.60 days were required for 4 mg/L KIN alone. Maximum 2.80 gm callus was also found with same treatment.

Effect of shoot proliferation with BA alone or in combination with NAA was observed in pineapple. Maximum 17.2 days to shoot induction was recorded in 4.0 mg/L BA whereas minimum 11 days in media containing 3.0 mg/L BA + 3.0 mg/L NAA from callus. The highest number of shoots (10.6 after 6 weeks of subculture) was recorded in 2.0 mg/L BA + 1.0 mg/L NAA and the lowest number of shoots (3.2 after 6 weeks of subculture) was in only 4.0 mg/L BA.

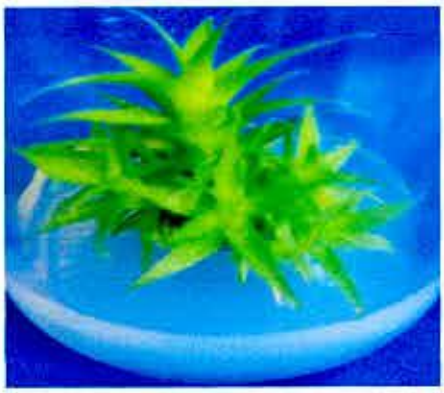
Effect of shoot proliferation with KIN alone or in combination with NAA or IBA was also studied to find best treatment for commercial production of pineapple plantlets. The results showed that the highest percentage (85%) of shoot induction was recorded with 3.0 mg/L KIN+1.0 mg/L NAA. But minimum 7.60 days required in media

containing 2.0 mg/L KIN + 1.5 mg/L NAA. The highest no. of shoot (18.40) was recorded in 3.0 mg/L KIN + 1.0 mg/L IBA and the lowest number of shoots (4.40) after 6 weeks of subculture in 1.0 mg/L KIN. Moreover, The longest shoot (9.04 cm after 6 weeks) was found in 3.0mg/L KIN+ 2.0 mg/L IBA and the lowest (6.30 cm) length of shoot in case of 4.0 mg/L KIN alone after 6 weeks of subculture. It indicates that combination of KIN and IBA performed better than only single treatment of hormone.

NAA or IBA alone, or combination of both was studied for root induction and found that the highest percentage (75%) of root induction was recorded with 2.0 mg/L IBA+ 1.5 mg/L NAA. The lowest percentage (20%) of root induction was recorded in media lack of plant growth regulator. The maximum 34.40 days to root induction was required in media devoid of growth regulator and the minimum 16 days was required in case of 2.0 mg/L IBA+1.5 mg/L NAA. But the highest number of roots (15.60 after 6 weeks) per explants was recorded in 3.0 mg/L IBA+ 1.0mg/L NAA rooting media. In addition, longest root (7 cm) was also found with 2.0 mg/L IBA+ 1.5 mg/L NAA.

Regenerated plantlets were acclimatized and it showed 85% survival in growth chamber conditions and 82% in shade house stage of hardening and 78% in open atmosphere at direct sunlight.

In conclusion, the protocol developed from the present study may be useful for large scale production of healthy, disease free planting material in pineapple. The results showed that combinations of KIN + NAA and KIN+IBA were better than all other hormones under study for shoot multiplication and IBA+NAA combination was good for root proliferation within short time. Regenerated plants were found to be morphologically similar to the mother plant. This protocol can be used for commercial cultivation of pineapple.



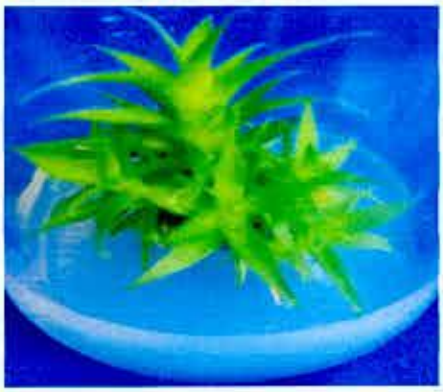
Chapter VI
Recomendations

CHAPTER VI

RECOMMANDATIONS

Following recommendations could be addressed based on the present experiment:

- Further study can be done with more variety of Pineapple.
- In addition to crown tip culture, sucker, slip, gil and leaf culture can be done.
- Callus induction can be done with 2.4-D or other callus induction hormone for large number of shoot regeneration.
- Research should be carried out upto pineapple fruit production and evaluate extant of genetic variation between tissue cultured plant and conventional method of Pineapple plant.



Chapter VII
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CHAPTER VII

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Chapter VIII
Appendices

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: Solubility and molecular weight of some hormones

Hormones (Solute	Molecular weight	Solvents used
BA	225.3	1 N NaOH
KIN	215.2	1 N NaOH
NAA	175.139	70% ethyl alcohol
IBA	203.2	70% ethyl alcohol

Appendix III: Analysis of variance on days to callus induction with KIN

Source	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability
Treatment	4	129.840	32.460	12.6797	0.0001
Error	20	40.960	2.560		
Total	24	170.800			
CV (%)	25.87				
$LSD_{(0.05)}$	2.145				

Appendix IV: Analysis of variance of weight of callus after 3 weeks with KIN

Source	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability
Treatment	4	7.524	1.814	503.7779	0.0001
Error	20	0.058	0.004		
Total	24	7.312			
CV (%)	5.62				
$LSD_{(0.05)}$	0.084				

Appendix V: Analysis of variance of weight of callus after 6 weeks with KIN

Source	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability
Treatment	4	4.550	1.137	117.8653	0.0001
Error	20	0.154	0.010		
Total	24	4.704			
CV (%)	5.83				
$LSD_{(0.05)}$	0.1341				

Appendix VI: Analysis of variance of days to callus induction with KIN+NAA

Source	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability
Treatment	13	361.600	27.815	23.5023	0.0001
Error	56	61.543	1.184		
Total	69	423.143			
CV (%)	4.50				
$LSD_{(0.05)}$	1.381				

Appendix VII: Analysis of variance of weight of callus after 3 weeks KIN+NAA

Source	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability
Treatment	13	16.848	1.296	292.2825	0.0001
Error	56	0.231	0.004		
Total	69	17.079			
CV (%)	4.21				
$LSD_{(0.05)}$	0.08027				

Appendix VIII: Analysis of variance of weight of callus after 6 weeks KIN+NAA

Source	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability
Treatment	13	12.940	0.995	108.1552	0.0001
Error	56	0.479	0.009		
Total	69	13.419			
CV (%)	4.4				
$LSD_{(0.05)}$	0.1204				

Appendix IX: Analysis of variance of days to shoot induction with BA

Source	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability
Treatment	4	232.000	58.00	178.4615	0.0001
Error	20	5.200	0.325		
Total	24	237.200			
CV (%)	3.17				
$LSD_{(0.05)}$	0.7643				

Appendix X: Analysis of variance of number of shoot/explants after 3 weeks with BA

Source	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability
Treatment	4	12.640	3.160	19.7500	0.0001
Error	20	2.560	0.160		
Total	24	15.20			
CV (%)	3.81				
$LSD_{(0.05)}$	0.5363				

Appendix XI: Analysis of variance of number of shoot/explants after 6 weeks with BA

Source	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability
Treatment	4	83.760	20.940	47.5909	0.0001
Error	20	7.040	0.440		
Total	24	84.20			
CV (%)	7.27				
$LSD_{(0.05)}$	0.8894				

Appendix XII: Analysis of variance of length of shoot after 3 weeks with BA

Source	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability
Treatment	4	7.002	1.750	179.0690	0.0001
Error	20	0.156	0.010		
Total	24				
CV (%)	2.95				
$LSD_{(0.05)}$	0.1341				

Appendix XIII: Analysis of variance of length of shoot after 6 weeks with BA

Source	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability
Treatment	4	26.454	6.613	94.4714	0.0001
Error	20	0.110	0.070		
Total	24	26.564			
CV (%)	1.62				
$LSD_{(0.05)}$	0.1122				

Appendix XIV: Analysis of variance of days to shoot induction with BA+NAA

Source	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability
Treatment	8	613.911	76.739	163.9525	0.0001
Error	36	14.978	0.468		
Total	44	628.889			
CV (%)	4.87				
$LSD_{(0.05)}$	0.88				

Appendix XV: Analysis of variance of no. of shoot/explants after 3 weeks with BA+NAA

Source	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability
Treatment	8	79.111	9.889	43.1515	0.0001
Error	36	7.333	0.229		
Total	44	86.444			
CV (%)	12.45				
$LSD_{(0.05)}$	0.61				

Appendix XVI: Analysis of variance of no. of shoot/explants after 6 weeks with BA+NAA

Source	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability
Treatment	8	291.600	36.450	128.6471	0.0001
Error	36	9.067	0.283		
Total	44	291.883			
CV (%)	7.13				
$LSD_{(0.05)}$	0.8894				

Appendix XVII: Analysis of variance of length of shoot after 3 weeks with BA+NAA

Source	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability
Treatment	8	23.986	2.998	59.96	0.0001
Error	36	0.158	0.050		
Total	44	24.144			
CV (%)	1.61				
$LSD_{(0.05)}$	0.1122				

Appendix XVIII: Analysis of variance of length of shoot after 6 weeks with BA+NAA

Source	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability
Treatment	8	52.857	6.607	273.2889	0.0001
Error	36	0.774	0.024		
Total	44	53.631			
CV (%)	2.61				
$LSD_{(0.05)}$	0.12				

Appendix XIX: Analysis of variance of days to shoot induction with KIN

Source	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability
Treatment	4	468.960	117.240	372.1905	0.0001
Error	20	5.040	0.315		
Total	24	474.0			
CV (%)	3.61				
$LSD_{(0.05)}$	0.75				

Appendix XX: Analysis of variance of no. of shoot/explants after 3 weeks with KIN

Source	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability
Treatment	4	53.360	13.340	95.2857	0.0001
Error	20	2.240	0.140		
Total	24	55.60			
CV (%)	12.64				
$LSD_{(0.05)}$	0.50				

Appendix XXI: Analysis of variance of no. of shoot/explants after 6 weeks with KIN

Source	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability
Treatment	4	192.400	48.100	213.7778	0.0001
Error	20	3.600	0.225		
Total	24	196.0			
CV (%)	8.18				
$LSD_{(0.05)}$	0.60				

Appendix XXII: Analysis of variance of length of shoot after 3 weeks with KIN

Source	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability
Treatment	4	11.889	2.972	304.1483	0.0001
Error	20	0.156	0.010		
Total	24	11.899			
CV (%)	2.68				
$LSD_{(0.05)}$	0.13				

Appendix XXIII: Analysis of variance of length of shoot after 6 weeks with KIN

Source	Degrees of Freedom	Sum of squares	Mean Square	F-value	Probability
Treatment	4	50.990	12.747	74.982	0.0001
Error	20	0.270	0.170		
Total	24				
CV (%)	2.19				
$LSD_{(0.05)}$	0.17				

Appendix XXIV: Analysis of variance of days to shoot induction of pineapple with KIN+NAA

Source	Degrees of Freedom	Sum of Squares	Mean Square	F-value	Probability
Treatment	8	1001.200	0.744	210.5327	0.0001
Error	36	19.022	125.150		
Total	44	1192.22			
CV (%)	6.93				
$LSD_{(0.05)}$	0.99				

Appendix XXV: Analysis of variance of no. of shoot after 3 weeks with KIN+NAA

Source	Degrees of Freedom	Sum of Squares	Mean Square	F-value	Probability
Treatment	8	342.178	42.772	180.0936	0.0001
Error	36	7.600	0.238		
Total	44	349.778			
CV (%)	6.94				
$LSD_{(0.05)}$	0.63				

Appendix XXVI: Analysis of variance of no. of shoot after 6 weeks with KIN+NAA

Source	Degrees of Freedom	Sum of Squares	Mean Square	F-value	Probability
Treatment	8	729.844	90.856	217.3289	0.0001
Error	36	13.378	0.418		
Total	44	740.222			
CV (%)	5.43				
$LSD_{(0.05)}$	0.83				

Appendix XXVII: Analysis of variance of length of shoot after 3 weeks with KIN+NAA

Source	Degrees of Freedom	Sum of Squares	Mean Square	F-value	Probability
Treatment	8	27.442	3.430	503.8577	0.0001
Error	36	0.218	0.007		
Total	44	27.660			
CV (%)	1.85				
$LSD_{(0.05)}$	0.11				

Appendix XXVIII: Analysis of variance of length of shoot after 6 weeks with KIN+NAA

Source	Degrees of Freedom	Sum of Squares	Mean Square	F-value	Probability
Treatment	8	86.850	10.856	97.2792	0.0001
Error	36	0.139	0.004		
Total	44	86.989			
CV (%)	2.97				
$LSD_{(0.05)}$	0.08				

Appendix XXIX: Analysis of variance of days to shoot induction with KIN+IBA

Source	Degrees of Freedom	Sum of Squares	Mean Square	F-value	Probability
Treatment	8	834.800	104.350	146.7422	0.0001
Error	36	22.756	0.711		
Total	44	857.556			
CV (%)	1.91				
$LSD_{(0.05)}$	1.09				

Appendix XXX: Analysis of variance of no. of shoot/explants after 3 weeks with KIN+IBA

Source	Degrees of Freedom	Sum of Squares	Mean Square	F-value	Probability
Treatment	8	450.880	56.350	222.9231	0.0001
Error	36	8.089	0.253		
Total	44	458.800			
CV (%)	5.63				
$LSD_{(0.05)}$	0.65				

Appendix XXXI: Analysis of variance of no. of shoot/explants after 6 weeks with KIN+IBA

Source	Degrees of Freedom	Sum of Squares	Mean Square	F-value	Probability
Treatment	8	880.800	110.100	129.5294	0.0001
Error	36	27.200	0.850		
Total	44	908.0			
CV (%)	7.28				
$LSD_{(0.05)}$	1.19				

Appendix XXXII: Analysis of variance of length of shoot after 3 weeks with KIN+IBA

Source	Degrees of Freedom	Sum of Squares	Mean Square	F-value	Probability
Treatment	8	76.151	9.519	95.19	0.0001
Error	36	0.315	0.100		
Total	44	76.416			
CV (%)	1.90				
$LSD_{(0.05)}$	0.13				

Appendix XXXIII: Analysis of variance of length of shoot after 6 weeks with KIN+IBA

Source	Degrees of Freedom	Sum of Squares	Mean Square	F-value	Probability
Treatment	8	116.883	14.610	73.05	0.0001
Error	36	0.063	0.200		
Total	44	116.746			
CV (%)	2.59				
$LSD_{(0.05)}$	0.08				

Appendix XXXIV: Analysis of variance of days to root induction with IBA

Source	Degrees of Freedom	Sum of Squares	Mean Square	F-value	Probability
Treatment	4	459.360	114.840	82.6187	0.0001
Error	20	22.240	1.390		
Total	24	481.60			
CV (%)	4.41				
$LSD_{(0.05)}$	1.13				

Appendix XXXV: Analysis of variance of no. of root/explants after 3 weeks with IBA

Source	Degrees of Freedom	Sum of Squares	Mean Square	F-value	Probability
Treatment	4	145.600	36.400	242.667	0.0001
Error	20	2.400	0.150		
Total	24	148.00			
CV (%)	12.91				
$LSD_{(0.05)}$	1.98				

Appendix XXXVI: Analysis of variance of no. of root/explants after 6 weeks with IBA

Source	Degrees of Freedom	Sum of Squares	Mean Square	F-value	Probability
Treatment	4	376.240	94.060	243.3117	0.0001
Error	20	6.160	0.386		
Total	24	382.400			
CV (%)	11.32				
$LSD_{(0.05)}$	0.50				

Appendix XXXVII: Analysis of variance of average length of root with IBA

Source	Degrees of Freedom	Sum of Squares	Mean Square	F-value	Probability
Treatment	4	70.954	17.738	84.4667	0.0001
Error	20	0.038	0.210		
Total	24	70.982			
CV (%)	1.95				
$LSD_{(0.05)}$	0.80				

Appendix XXXVIII: Analysis of variance of days to root induction with NAA

Source	Degrees of Freedom	Sum of Squares	Mean Square	F-value	Probability
Treatment	4	386.560	96.640	53.9888	0.0001
Error	20	28.640	1.790		
Total	24	415.200			
CV (%)	4.93				
$LSD_{(0.05)}$	1.13				

Appendix XXXIX: Analysis of variance of no. of root/explants after 3 weeks with NAA

Source	Degrees of Freedom	Sum of Squares	Mean Square	F-value	Probability
Treatment	4	77.040	19.1290	62.1290	0.0001
Error	20	4.960	0.310		
Total	24	82.000			
CV (%)	22.45				
$LSD_{(0.05)}$	0.72				

Appendix XL: Analysis of variance of no. of root/explant after 6 weeks with NAA

Source	Degrees of Freedom	Sum of Squares	Mean Square	F-value	Probability
Treatment	4	202.960	50.740	79.2812	0.0001
Error	20	10.240	0.640		
Total	24	213.200			
CV (%)	16.13				
$LSD_{(0.05)}$	1.03				

Appendix XLI: Analysis of variance of length of root after 6 weeks with NAA

Source	Degrees of Freedom	Sum of Squares	Mean Square	F-value	Probability
Treatment	4	73.904	18.476	616.8667	0.0001
Error	20	0.044	0.030		
Total	24	73.944			
CV (%)	1.57				
$LSD_{(0.05)}$	0.07				

Appendix XLII: Analysis of variance of days to root induction with NAA+IBA

Source	Degrees of Freedom	Sum of Squares	Mean Square	F-value	Probability
Treatment	6	1165.771	189.295	150.2911	0.0001
Error	28	30.229	1.260		
Total	34	1166.0			
CV (%)	5.11				
$LSD_{(0.05)}$	1.47				

Appendix XLIII: Analysis of variance of no. of root/explants with NAA+IBA after 3 weeks

Source	Degrees of Freedom	Sum of Squares	Mean Square	F-value	Probability
Treatment	6	283.200	47.200	120.8780	0.0001
Error	28	9.371	0.390		
Total	34	192.571			
CV (%)	13.02				
$LSD_{(0.05)}$	0.82				

Appendix XLIV: Analysis of variance of no. of root/explants with NAA+IBA after 6 weeks

Source	Degrees of Freedom	Sum of Squares	Mean Square	F-value	Probability
Treatment	6	749.371	124.895	158.4773	0.0001
Error	28	18.914	0.788		
Total	34	768.285			
CV (%)	10.86				
$LSD_{(0.05)}$	1.16				

Appendix XLV: Analysis of variance of length of root with NAA+IBA after 6 weeks

Source	Degrees of Freedom	Sum of Squares	Mean Square	F-value	Probability
Treatment	6	168.832	28.139	127.8825	0.0001
Error	28	0.039	0.002		
Total	34	186.871			
CV (%)	3.77				
$LSD_{(0.05)}$	0.06				