Optimization of Protocol for In Vitro Regeneration of Selected Cultivars of Banana

By

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THE MOST BENIFICENT

Department of Bioinformatics and Biotechnology

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Dated: 03-Feb-2016

FINAL APPROVAL

It is certificate that we have read the thesis submitted by Mr. Arslan Bashir and it is our judgment that this project is of sufficient standard to warrant its acceptance by the International Islamic University, Islamabad for the M.S Degree in Biotechnology.

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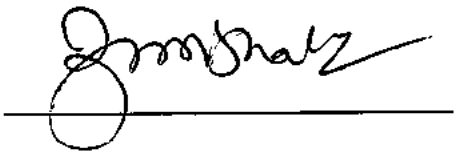

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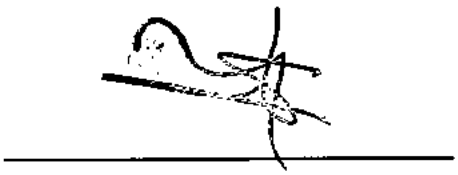

A thesis submitted to Department of Bioinformatics and Biotechnology Faculty of Basic and applied Sciences, International Islamic University, Islamabad as a partial fulfillment of requirement for the award of the degree of MS Biotechnology.

DEDICATION

This thesis is dedicated to my beloved parents whose hands always rise in prayers for my success. Their prayers always helped me in coming out from the complex situations.

DECLARATION

I hereby declare that the work present in the following thesis "Optimization of Protocol for *In Vitro* Regeneration of Selected Cultivars of Banana" is my own effort, except where otherwise acknowledged and that the thesis is my own composition. No part of the thesis has been previously presented for any other degree.

Date _____

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Arslan Bashir

LIST OF ABBREVIATIONS

Abbreviations	Complete Words
µM	Micro Moles
BAP	Benzyl Amino Purine
BBMV	Banana Bract Mosaic Virus
BBTV	Banana Bunchy Top Virus
BSV	Banana Streak Virus
cm	Centimeter
DW	Distilled Water
H_2SO_4	Sulphuric Acid
IAA	Indole-3-acetic acid
IBA	Indole-3-Butaric Acid
KNO_3	Potassium Nitrate
LSD	Least Significant Difference
M	Molar
mg/g	Milligram/Gram
mg/l	Milligram/Litre
MS	Murashige and Skoog
N	Normal
NAA	Naphthalene Acetic Acid
NARC	National Agriculture Research Center
PARC	Pakistan Agriculture Research Council
PGR	Plant Growth Regulators
PGRI	Plant Genetic Resource Institute
Ppm	Parts per million
SA	Salicylic Acid
TCT	Tissue Culture Technique
TR4	Topical Race 4

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Abstract

Banana is herbaceous, monocotyledonous and evergreen perennial plant. Most of the banana cultivars are susceptible to Banana Bunchy Top Virus (BBTV) and it causes significant economic loss to banana production. Micropropagation is preferred over the conventional method of propagation in banana due to faster multiplication rate, uniformity in planting materials and production of disease-free planting materials. The present study comprises of three exotic banana genotypes viz: Wiallium-8818 hybrid (Chinese variety), Pisang (Chinese variety) and Brazilian (Brazilian variety). Different combinations of IAA, NAA and BAP along with MS were prepared for optimizing tissue culture protocol. The Clorox at 50% concentration for 15 min gave minimum percentages for both fungal and bacterial contamination, and optimum survival percentage. Seven different combinations (T0-T6) of BAP and IAA and five combinations (RT0-RT4) of NAA and IAA were used to optimize multiplication and rooting protocols. It was found that BAP and IAA at 2.5 mg/L and 1 mg/L respectively (T5) gave maximum (6.53) mean number of shoots per explants while longest shoot length (7.14cm) was recorded at MS with BAP and IAA at 1 mg/L and 1 mg/L (T4) respectively. Maximum mean fresh weight value (12.96g) was recorded in MS medium supplemented with BAP and IAA at 5.0 mg/L and 1.0 mg/L (T6) respectively. The genotype Wiallum-8818 hybrid gained maximum value for mean number of shoots per explants (4.94) and longest shoot length (8.52cm) while the genotype Pisang gained maximum fresh weight value (8.95g). It was found that NAA at 0.5 mg/L (RT4) gave maximum (5.70) leaves per shoot while mean roots per shoot (4.67) was recorded at MS with 1mg/L NAA and 1mg/L IAA (RT3) . Maximum mean value (4.67) for roots per shoot was recorded in MS medium supplemented with 1mg/L NAA and 1mg/L IAA (RT3) . Maximum mean value (5.58cm) for longest root length was recorded in MS medium

supplemented with 1mg/L NAA and 0.5mg/L IAA (RT2) respectively. The genotype Wiallum-8818 hybrid gained maximum value for mean leaves per shoot (4.47) while variety Pisang showed maximum value for roots per shoot (4.11) and longest root length (5.48cm).

Chapter 1

Introduction

Chapter 1

INTRODUCTION

1.1 General introduction

The word banana is derived from Arabic language word 'banan' means fingers which probably being first used in Guinea (West Africa) with fruit introduction by Portuguese from where it spread to new world (Cheesman, 1948). The genus Musa is a member of Musaceae family which includes two other genuses viz: Ensete and Musella (Constantine and Rossel, 2001). All the three monocotyledons genera are technically defined as herbs.

1.2 Centre of origin, diversity and domestication

The generally accepted theory on origin of edible banana is that Malesia which includes regions of Malay, Indonesia, Philippines and New Guinea as primary centre while India as secondary centre of origin (Daniells *et al.*, 2001). It is believed that banana is originated through complicated hybridization process somewhere in modern Malaysia (Novak, 1992). Cultivated banana has triploid genome and believed to originate from two diploid species viz: *Musa acuminata* from Malaysia and *Musa balbsiana* from India (Georget *et al.*, 2000).

The two main secondary centers for *Musa* species are East Africa and West Africa. Polynesia is thought to be another secondary centre of banana origin due to long history of cultivation for more than 4000 years ago (De Langhe, 1995).

1.3 The banana plant

Banana is herbaceous, monocotyledonous and evergreen perennial plant. It is herbaceous due to absence of woody component and perennial as new suckers grow up from base of mother plant. Banana male and female flower can only morphologically distinguishable when the young inflorescences borne about 150 cm up the pseudo stem and about 12 cm long. The male flower have abortive ovary while female flower have well-developed ovary. Edible pulp develops from ovary wall (Daniells *et al.*, 2001).

1.4 Soil and climate

Deep well drained loamy soils are ideal for banana cultivation (Stover and Simmond, 1987). Due to restricted root zone, two most important considerations in soil selection for banana cultivation are adequate depth and drainage. Alkaline soils response well while saline soils exceeding 0.05% salinity are not suitable for banana cultivation (Ikisan, 2000).

Banana is well adapted to wide range of climatic conditions from wet tropics to dry subtropical zones. It grows well in temperature range of 15-40°C. The two important climatic limiting factors are incidence of frost and storms or cyclones which restrict its cultivation to cooler regions. Banana need 10mm monthly rainfall for proper growth on average(Ikisan, 2000).

1.5 World banana production and market trends

The world banana production got a major rise of 29.5 million tons from 2000 to 2011 (figure 1.1). In 2013-14 the global banana trade reached a new peak (≤17 million tonnes) due to

production recovery in the major banana-producing areas and strong demand in all major markets. This expansion has been driven by increased shipments from Latin America and the Caribbean, where in 2013-14 supplies grew by 829000 tonnes. Ecuador, the largest exporter worldwide, experienced a slump in exports in 2012-13 due to floods, but rebounded in 2013-14, supplying 5.3 million tonnes of bananas to world markets.

India, the largest banana producer in the world, has been rapidly increasing cultivated area and volumes of production over the past decade. Although India currently produces predominantly for the domestic market, it also supplies bananas to markets in Nepal and the Middle East – total exports equaled 36 000 tonnes in 2013-14. The three largest importers in the world are European Union, United States and Russian Federation. The largest importers in Asia are China and Japan. China's own production has been growing rapidly over the past years, satisfying a larger share of the demand. In China, the second largest banana producer in the world after India, an estimated 40,000 hectares are now affected in varying levels, causing significant damages, although no reliable data exist on the volume of lost production. India, world's largest producer, is not yet affected by TR4, although a race 1 strain has already been reported to cause infection on Cavendish bananas. Being close to China, India is particularly vulnerable to the risk of TR4 spread (FAO, 2015).

1.6 Major threat to banana in Pakistan

The banana crop was first threatened with an unknown disease observed in Thatta district of Sindh province during year 1988. It was in July 1991, the proper diagnosis of diseased field

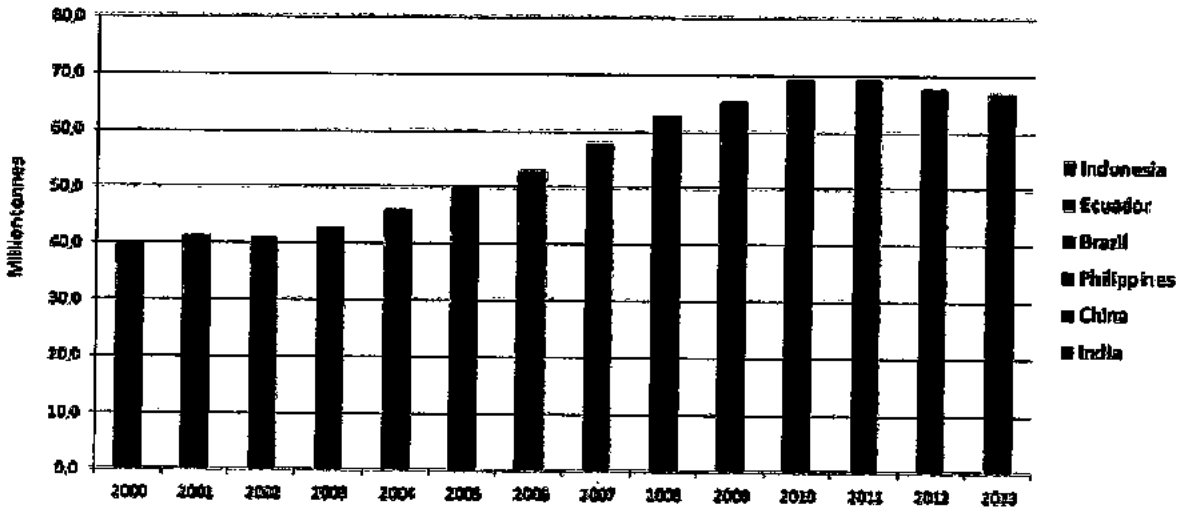


Figure 1.1: World banana production for the year 2000-13 (FAO 2015).

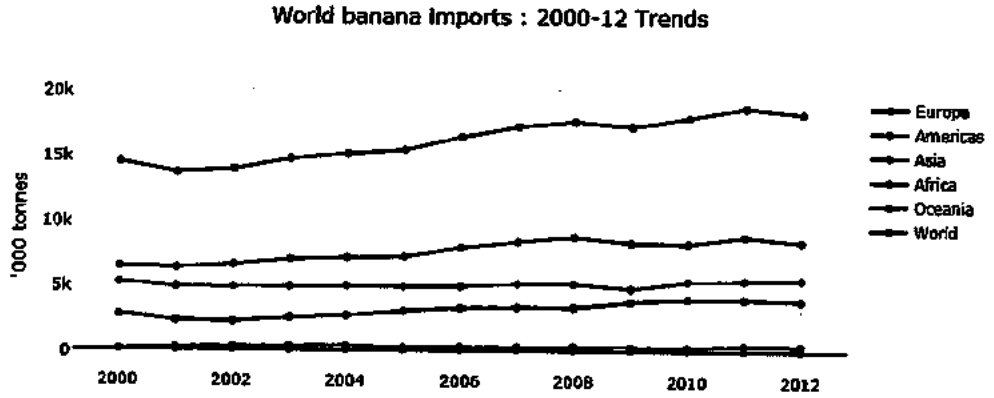


Figure 1.2: World banana import trends for year 2000-12 (FAO 2015).

revealed that this disease is due to banana bunchy top virus. The disease spread throughout the province due to non availability of virus free plants across the planting area (PARC, 2004) and consequently the total area under banana decreased from 23,500 ha to 11,200 ha. The total banana production decreaed from 209,800 tons to 44,200 tonnes which increased the demand and price of banana as a result farmers started to grow banana in areas where banana was not previously grown (Khalid and Soomro, 1993). This results in spread of disease to areas where it was not previously reported. Although an increase in total area under banana cultivation was observed in 1997-98 (26,000 ha), but total production remained low due to disease plantation and unavailability of virus free planting material.

1.7 Banana bunchy top virus

Banana Bunchy Top Virus (BBTV) is caused by single stranded DNA virus with isometric virons 18-20 nm in diameter. The destructive virus caused huge losses to banana crop in Tropical Asia, South Pacific and Australia. Most of the banana cultivars are susceptible to BBTV and it causes significant economic losses to banana production (Dale, 1987). BBTV has become a serious epidemic disease in several Asian countries including India and Pakistan.

The word 'bunchy top' is given to disease on account of observed visual symptoms of virus attack on banana. The affected leaves are more upright than usual, pale yellow leaf margins, dark green flecks along the vein producing dot and dashes pattern, waxy leaf margins and chocking of emerging leaves giving a 'bunchy top' appearance are some of the visual symptoms of BBTV. All suckers from BBTV infected mother plant will be diseased. The vector of BBTV disease is aphid *Pentalonia nigronervosa* (Harding *et al.*, 1993).

The control of BBTV is positively correlated with destruction of its vector, aphids. Many commercial insecticides are available to control aphids. Polygodial at 50g/ha (Asakawa *et al.*, 1988), Azadirachtin >0.5% concentration (West and Mordue, 1992) and Alarm pheromones, combination of organic phosphate and *b*-faraneses (Ester *et al.*, 1993) are reported chemical control of black aphids. The other major viral attacks on banana crop include Banana Streak Virus (BSV) and Banana Bract Mosaic Virus (BBMV).

Conventionally banana is vegetative propagated crop through suckers. Therefore the progeny of virally infected mother plant will also diseased. The possible solution is use of tissue culture technology which equips the plant biotechnologist to rapidly clone disease free elite germplasm, independent of climatic conditions, within the limited timeframe and space. In order to provide disease free planting material, scientists are working to develop protocols generally for all economically important crops and specifically for each genotype.

1.8 Plant tissue culture

The scientific basis of plant tissue culture is concept of totipotency. This concept states that the genetic potential to develop into a single plant is available in every living cell. The whole process begins with excision of meristematic part followed by sterilizing and placing on sterilized artificial nutrient medium. To complete the process successfully, organic salts (analytical grade) as normal growth elements, sugar as carbon source, vitamins as essential elements and growth hormones are needed in specified quantity for each crop. Agar-agar, gellan gum and gelrite are some of the commercially available solidifying agents and usually added to support the plants. Some micropropagation protocols are based on liquid media in which flasks

are continuously shake which subsequently provides the aerations by reciprocating the plant tissue.

Although micro propagation is promising solution to clone rapidly elite genotypes but the process needs special attention to tackle problems which could hinder the success of tissue culture process including oxidative browning of the wounded tissues and low number of shoots produce per explants (Ngomuo *et al.*, 2014).

1.9 Advantages of micropropagation over conventional propagation

- It is a rapid clonal multiplication tool and introduction can be bulked up to enlarge quantity for simultaneously released (George and Sherrington, 1994).
- It is independent of climatic condition and round year production is feasible due to environmentally controlled conditions (George and Sherrington, 1994).
- The aseptic planting material is exchangeable between countries (George Sherrington, 1994).
- It can bring about heritable variation which could be further exploit in breeding programs (George and Sherrington, 1994).

1.10 Banana tissue culture

Micropropagation is preferred over the conventional method of propagation in banana due to faster multiplication rate, uniformity in planting materials, production of disease-free planting materials, higher bunch weight, more fingers and hands and less variability in fruit size

and shape. The apical meristem or shoot-tip culture is very efficient for rapid clonal Micropropagation (Lalrinsanga *et al.*, 2013).

Banana shoot-tip cultures were most suitable for micropropagation for large scale production. Tissue culture has become a highly useful technique in the field of agriculture (Jaisy and Ghai, 2011). The explants started to show the signs of shoot proliferation after a weak of culturing (Govindaraju *et al.*, 2012).

1.11 Objectives

The objectives of the present study are listed below

- To optimize level of different plant hormones (BAP and IAA) on different morphological traits of banana multiplication stage.
- To optimize level of different auxins (IAA and NAA) for *in vitro* rooting of exotic banana cultivars.
- Production of virus (BBTV) free banana plants.
- To carry out rapid multiplication of banana plants through plant tissue culture technique.

Chapter 2

Review of Literature

Chapter 2

REVIEW OF LITERATURE

In an experiment to optimize micropropagation protocols MS medium was supplemented with different concentrations and combinations of benzylaminopurine (BAP) (0, 2, 4, 6 mg L-1) and indole acetic acid (IAA) (0.5 and 1.0 mg L-1). Exotic banana genotype GCTCV-215 (AAA), Yangambi Km-5 (AAA) and FHIA-23 (AAAA) were used in research work. Significant ($p \leq 0.05$) variation was observed for varieties, treatments and varieties x treatment for all the parameters viz: days for bud initiation, rate of shoot proliferation (%), number of multiple shoots, shoot length (cm) and fresh mass of shoot (g). Synergistic effects of BAP and IAA were observed in both genotypes GCTCV-215 and Yangambi Km-5. MS medium supplemented with BAP (4.0 mg/L) with IAA (1.0 mg/L), showed maximum shoots and fresh mass of shoot in short period of time for GCTCV-215 and Yangambi Km-5. *In vitro* plantlets were shifted from growth room to green house in polythene bags containing garden soil and humus mixture in 1:1 ratio (Qamar *et al.*, 2015).

After 20-25 days of culture initiation, meristem grew into a green globular hard coat mass and adventitious plantlets were developed. Among the different concentrations BAP (5 mg/L) and NAA (0.5 mg/L) showed highest proliferation of shoots per explant (1.00, 1.5, 1.75 and 3.17), longest shoot length (0.42, 2.34, 2.64 and 3.46 cm) and maximum number of leaves (2.33, 3.00, 3.33 and 4.33) at 10, 20, 30 and 60 days respectively (Gebeyehu, 2015).

The following study was carried out with a purpose of establishing a standard method or protocol for the *in vitro* plan propagation or multiplication from the excision of shoot tips of banana. The BAP and IBA was used on the medium of MS with different concentration 0.0, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0 and 7.0 mg/L and (0.0, 1.0, 2.0, 3.0, 4.0 and 5.0 mg/L respectively. The purpose was to check the effect of *in vitro* shoot regeneration and root formation of different varieties of banana from Amritasagar and Sabri cultivars. The highest level of shoot formation was seen in the media 0.5 mg/L BAP as 50.0% and 30.0% shoot formation and the number of single shoots were 3.50 and 2.00cm and the longest shoot was 2.64 cm and 2.16 cm . on the other hand the study of root formation was also observed using the basal medium containing 0.3 mg/L IBA in Amritasagar and Sabri respectively and the maximum results of number of roots were 3.83 and 2.50 and the highest root was 3.60 cm and 3.10 cm. under the conditions of ex vitro it was observed that both cultivars survived at the rate of more than 82%.so it was concluded from the given study that 0.5 mg/L Benzyle Amino Purine (BAP) and 0.3 mg/L of IBA along with the MS media are suitable for shoot and root formation of Amritasagar and Sabri banana cultivars respectively using shoot tip culture technique (Ferdous *et al.*, 2015).

In another similar study of *Musa acuminate L* cv. Cavandish Dwarf ,the *in vitro* propagation was carried out by using some simple and effective compositions of different medium and the purpose of the study was to develop some successful micro propagation methods for the propagation of banana cultivar. The basal medium used in this study was MS and it was additionally supplemented with different concentration of BAP for induction of shoot and the concentration of 2 mg L-1 BAP gave the best results. The different concentrations of Kn along with BAP and MS was added for the formation of multiple shoots and the best results were obtained in the ½ of MS medium that was supplemented with 2.0mg L-1 BAP and 1.0mg L-1

Kn. The rooting of micro shoots they cultured on ½ MS medium along with additional Concentration of IBA and the best results of rooting were seen in 1/2 MS medium containing the IBA at the level of 0.5mg L (Devendrakumar *et al.*, 2013).

Highest multiple shoot induction was (2.17 shoots) was observed in MS medium supplemented with BAP (5mg/L) while the longest regenerated shoots after 45 days of incubation was resulted on MS with NAA (1 mg/L) and BAP (0.2 mg/L). NAA (2mg/L) gave maximum number of roots. The mixture of sand and FYM (2:1), and soil and vermicompost (2:1) gave maximum (98-100 %) hardening survival percentages (Lalrinsanga *et al.*, 2013).

Liquid MS supplemented with BAP (5.0mg/L) and IAA (1.0mg/L) along with 10 % coconut water (CW) gave maximum shoot proliferation in micropropagation of banana (*Musa sapientum L.*) after 15 days of culturing. Rooting was successfully achieved on full strength MS along with IAA (2 mg/L) (Iqbal *et al.*, 2013).

Using sword suckers the invitro micropropagation techniques were developed for banana *Musa sapientium* (Cavendish Dwarf). The most appropriate method found during this experiment was the combination of 4mg/L BAP and 0.2mg/L NAA along with Murashige and Skoog's basal medium.The observations were recorded separately for all five subcultures at an interval of four weeks. The concentration of hormones in E culture was increased by 1mg/L i.e. (BAP-5mg/L + NAA-0.3mg/L) for further multiplication. The shoots were excised for further and were transferred to another growth medium containing the hormone (NAA-1.5mg/L) and activated charcoal. After the rooting of the desired plants they were then shifted to primary and secondary hardening and then were transferred to a greenhouse for their proper growth.These hardened plants have been successfully established in soil (Ramachandran and Amutha, 2013).

In another similar study (Sipen and Davey,2012) reported that the BAP, IAA and Murashige and Skoog based medium were used to check their effects on shoot propagation and other plant's parameter of the growth like meristem, nodule and plants regeneration of the different varieties of Pisang Mas, Pisang Awak, Pisang Nangka, Pisang Berangan, Malaysian banana cultivars. Different combination of media were tested such as BAP at the concentration of 1mg/L to 14 mg/L with the concentration of 0.2 mg /L IAA on the other hand BAP was used with the concentration of 7mg/L to 14 mg/L along the same concentration of Indole Acetic acid (IAA). These combinations were used to evaluate shoot regeneration from the shoot tips and the proliferation and multiplication of nodule like meristems from the plant's scalps respectively. The regeneration of plant from their scalps were achieved using the BAP with the concentration of 1 mg/L and IAA with concentration of 0.2 mg/L separately and they were also used in the combination of both the growth hormones. After thirty days of culturing data was collected separately for the multiplication of shoot, proliferation or regeneration of nodule like meristems. In the following experimental trial the maximum of 5 shoots from one original shoot tip was achieved using the media supplied with BAP at the concentration of 5 mg/L in Pisang Nangka , 6 mg/L in Pisang Mas and Pisang Berangan and 7 mg/L in Pisang Awak and with the concentration of 0.2 mg/L of Indole Acetic acid (IAA). The result indicated that 11 mg /L of BAP with IAA 0.2 mg/L cause the maximum proliferation of nodule like meristems in the four different banana cultivars. Plant regeneration from scalps was optimum in all cases on medium containing 1 mg L^{-1} BAP and 0.2 mg L^{-1} IAA. After this study it was concluded that it is the first report that indicates the successful induction of maximum rate of proliferation of nodule like meristem and the regeneration of plant using the different scalps from the different varieties of

banana cultivars from Malaysian ,Pisang Nangka Pisang Mas, Pisang Berangan and Pisang Nangka.

MS with BAP (2.0 mg/L) showed maximum (80%) shoot proliferation while maximum percent of adventitious root formation was observed in half strength MS medium supplemented with 1.5mg/L Indole butyric acid (IBA) and 1.0mg/L Napthalene acetic acid (NAA). 75% survival efficiency was recorded in mixture (1:1) of sand and soil (Govindaraju *et al.*, 2012).

The frequency of bud formation increases proportionally with increase of BAP and maximum mean value (4.5 shoots per explant) was obtained on 7mg/L BAP concentration, however, further increase in BAP significantly decreases (3.2 shoots per explant) shoot bud formation for cultivar Basrai (Bhosale *et al.*, 2011).

Ali *et al.*, (2011) investigated the techniques for the *In vitro* plant propagations. In this study explants from the plants grown in the field were taken. Like the other similar studies they also used the MS, BAP, IAA and Kin supplemented medium in their experiments. The apical meristems of shoot with different sizes were cultured on (MS) medium that was additionally supplied with various concentrations and combinations of BAP (6-benzylamino-purine), Kin (kinetin) and NAA (α- naphthaleneacetic acid) either separately or combined with each other under different conditions of temperature ranged from 23 - 27°C. The response for the shoot formation using the apical meristem of shoot indicated that MS medium supplemented with 1.0 mg/L of BAP showed best results for the formation of shoot. For the multiplication of shoot, MS medium in combination of BAP and Kin as 1.0 mg/L of BAP + 0.25 mg/L of kin showed the best propagation and multiplication response. Within 21.5 days after the inoculation in shoot proliferation medium it gave almost 8 shoot per culture. The variation in temperatures also

affects the Shoot formation and multiplication/ proliferation. At 27°C ± 1°Cthe best results were observed. The rate of *in vitro* propagation was also varied in fact decreases with the increase or decrease of temperature. After 6-8 days of inoculation of shoots into rooting medium, for the best developed results of rooting the medium was supplemented with the additional IBA with the concentration of with the concentration of 1.0 mg/L plus NAA with the concentration of 0.5 mg/L. the results indicated that NAA+IBA showed per plant 3. Roots and an average root length observed was 2.4cm. After 21 days transplantation of peat moss in the glass house 100% hardening response was obtained.

In another similar study Jafari *et al.,* (2011) investigated that during the initiation stage of of rooting of *Musa acuminate* cv the bud formation in the shoot cultures were increased proportionally at the rate of concentrations of MS and BAP media 11,22 and 33 µM respectively. It was also observed that even the abnormal shoots formation was increased simultaneously by the use of highest level of BAP (33 µM). When the apical bud appeared the MS medium was provided with the lower concentrations or even without IAA (Indole Acetic Acid). The result of this study indicated that multiplication and elongation was enhanced by using proliferation media provided with IAA but it was unable to reduce the abnormality index that has occurred.

In optimizing regeneration protocol for banana cv. BARI Banana-I using MS medium with different combinations of cytokinin BAP and auxin NAA the highest shoot proliferation rate of (0.75, 2.75 and 6.25), longest shoot length (1.03, 2.45 and 3.38 cm), maximum number of leaves (2.50, 3.25 and 7.00 leaves/explants) and longest leaves (0.85, 2.70 and 4.23 cm) after 10, 20 and 30 days respectively, was obtained in MS medium supplemented with BAP (7.5 mg/L) and NAA (0.5 mg/L). The highest length of roots (2.93, 4.63 and 5.88 cm) recorded after 10, 20

and 30 days and maximum number of roots was obtained in MS medium with IAA (0.5 mg/L) and IBA (0.5 mg/L). Successful hardening was obtained in soil and cow dung (1:1) with over 95 percent survival rate (Al-amin *et al.*, 2009).

Rahman *et al.,* (2005) reported that shoot multiplication of banana cv. Anupomand the effects of different growth hormones as a single or in combinations were studied. Cytokinins that included BAP and KIN, auxins included IBA, NAA and IAA and coconut were studied. The multiplication rate varied in all these different treatments. The highest results were observed in the MS medium that was supplemented with 5mg/L of BAP amd KIN each separately along with 13% coconut water and the highest number of roots/shoots were 5.8±0.154. The response and survivability of shoots for rooting was highest with IBA than that of NAA and IAA hormones. The highest number of roots produced per shoot was 7±0.24 using MS medium supplemented with 1mg/L of IBA. Without the addition of any auxins in the medium all the roots/shoots grown were in less quantity like 2.60±0.219.The planlets that rooted well were transferred in the polybags and were successfully established in the soil.

In another study of banana multiplication and rooting the effect of different growth hormones like auxins and cytokinins were investigated on three types of newly selected superior bananas. The medium used for the rooting at the propagation stage was BAP (Benzylaminopurine) at the concentration of 5, 10, 20 and 30 µM and TDZ (Thidiazuron) at the concentration of 0.4, 1, 2 and 3 µM along with IAA (Indoleacetic acid). The medium used was compared with some other basal medium for the rooting they included active charcoal 5g/L, Indole-3-butyric acid (IBA), Murashige and Skoog (MS) and Naphtelene Acetic Acid (NAA). After the comparison of these medium the result indicated that TDZ and BAP gave the highest

elongation and in all three types of selected banana. Furthermore it was observed that proliferation and elongation was increased using combination of hormones cytokinin and Indole Acetic Acid than they used separately. Shoot proleferatuon was not increased by the concentration of Benzylaminopurine (BAP) below 20 µM or Thidiazuron (TDZ) while BAP with high concentration over 20 µM and TDZ over 2 µM reduced or suppressed the shoot elongation and proliferation. It was proved in the experiment that Charcoal alone gave the better results for rooting than the other growth hormone treatment including auxin and MS medium alone. At the end it was concluded that the best combination for the *in vitro* propagation of three types of banana includes 2 µMThidiazuron, 1 µM of Indoleacetic acid and 20 µM of Benzylaminopurine on MS medium supplemented with 5 g/L of charcoal at the rooting (Hamide and Pekmezcu, 2004).

In vitro multiplication techniques were reported in another research of banana cultivar. In this study it was observed that the BAP (Benzyle Amino purine) is necessary for the shoot multiplication of the banana cultivar plant. The 5mg/L BAP gave the maximum (4 to 5) plantlets. In the given study it was also observed that kinetin(2mg/L) is also very important for shooting and it increased the shoot elongation. The addition of 100 mg/L tyrosine controlled the secretions of phenolic compunds (Malik *et al.*, 2000)

Hirimburegama and Gamage, (1996) investigated that an alternative method called Invitro shoot-tip culture is a suitable method to that of traditional propagation of the Banana Musa Sp Cv. Cultivar. They tested *In vitro* multiplication on ten bananas Cultivar taken as a group AAA, AAB and ABB. The study indicated that the alternative shoot-tip culture technique is most suitable for the multiplication or propagation of local banana cultivars. The multiplication rate varied among the different groups and variation were also seen within the

same group of cultivars. The group AAA(Binkehel) showed the highest multiplication rate while the lowest rate of multiplication was observed in the other two group ABB (Alukehel) and AAB (Suwandel). Thus the study indicates that the rate of multiplication is dependent on cultivar and it was also observed that shoot multiplication is enhanced by sub culturing and the second subculture especially enhanced it at highest rate.

Chapter 3

Material and Methods

Chapter 3

MATERIAL AND METHODS

The present study was conducted in *In vitro* laboratory working under Plant Genetic Resource Institute (PGRI) National Agriculture Research Center (NARC) Islamabad.

3.1 Collection of explants

The experimental material consists of three banana varieties viz: 8818-William (Chinese), Pisang (Chinese) and Brazilian (Brazilian). The suckers were collected from the banana fields of National Agriculture Research Center (NARC) at Thatta, Sindh. The sword like suckers having broad base, vigorous growth and visually free of any pest or disease infestation were selected. Soil around the selected sucker was carefully removed with help of spade so that the root portion is exposed. The next step is removal of sucker from mother plant and is subjected to care and expertise so that the mother plant and sucker being removed may not be damaged. This mentioned step was performed with sharp cutter. The cutter was sterilized using rectified spirit each time before being used for individual sucker. Roots were cut to detach the suckers from mother plant. The leaves of suckers were removed and each sucker was put in separate polythene bag and tagged.

3.2 Preparation and sterilization of explants

Individual suckers were washed in running tap water for 30 minutes to remove mud and debris attached at the base. The pseudo stem was trimmed vertically and horizontally to expose

the inner most shoot with help of knife and cutter. The dissecting instrument was partially sterilized periodically with rectified spirit. Before sterilization the length and width of explants were 3-5 cm and 2-4 cm respectively. After this step explants were subjected to sterilization.

The sterilization was performed in Laminar Flow Hood using different concentrations (0-50%) of commercial bleach (Clorox, Sodium Hypo chloride) solution having 1-2 drops of tween 20 (sigma) prepared in autoclaved distilled water and subjected to different time interval (0-15 minutes) as given in table 3.1. After Clorox treatments , explants were washed for three times with autoclaved distilled water for ten minutes each. The meristem along with shoot primordia was aseptically excised with help of autoclaved scalpel and forceps and final size was kept around 2-3 cm high and 1-2 cm wide followed by culturing on MS medium supplemented with 8 milligram per liter 6-benzyl amino purine (BAP) and 1 milligram 3-indole acetic acid (IAA).

3.3 Preparation of Media

The media preparation is an important step in plant tissue culture experiments and requires high level accuracy and expertise. The medium is usually prepared with help of stock solutions, prepared in advance. This helps to avoid inaccuracy and makes easy to vary calculations. The following details methodology was adopted during media preparation.

3.3.1 Preparation of stock solution

All Murashige and Skoog (MS) stock solutions were prepared using distilled water alone while stocks solutions of Plant Growth Regulators (PGR's) were prepared using ethanol or 1N NaOH as dissolvent and distilled water as diluting agent. These stock solutions

Table 3.1 Different concentration of Clorox against different time intervals for optimization of sterilization protocol

Clorox concentration	Time
0%	5mins
	10mins
	15mins
10%	5mins
	10mins
	15mins
25%	5mins
	10mins
	15mins
50%	5mins
	10mins
	15mins

were stored in refrigerator at 4°C subjected to future use. The detailed of each type of stock solution is given below.

3.3.1.1 Murashige and Skoog (MS) macro nutrients stock solution

Following inorganic salts viz: ammonium nitrate, potassium nitrate, calcium chloride, magnesium sulphate and dihydrogen potassium phosphate (Sigma Aldrich, USA) were used to prepared the stock solution of macro nutrients. The stock solution of macro nutrients was prepared by dividing five salts into three stock solutions viz.: A, B and C. Individual salts were carefully weighed on electronic balance. Stock A consists of ammonium nitrate, potassium nitrate and calcium chloride. Eight hundred milliliter of distilled water was poured in glass beakers and placed on magnetic stirrer and proper time was given to dissolve individual salt completely before adding next salt. After dissolving all salts volume of stock was made up to one liter in glass cylinder.

Similarly stock B and C were prepared by dissolving magnesium sulphate and potassium di hydrogen phosphate respectively. The relative mass of each type of inorganic salt is given in Table 3.2.

3.3.1.2 Murashige and Skoog (MS) micro nutrients stock solution

The stock of micro nutrients were prepared using following inorganic salts viz: manganese sulphate, boric acid, potassium iodide, copper sulphate, sodium molybodate and cobalt chloride (Sigma Aldrich, USA). Similar methodology as described in 3.3.1.1 was adopted.

3.3.1.3 Stock solution for sodium iron salt

Sodium iron salt can be prepared either by using 1.84 g/L of sodium iron ethylene diamine tetra acetic acid (FeNaEDTA) or by sodium ethylene diamine tetra acetic acid (NaEDTA) and ferrus sulphate ($FeSO_4$) at concentration of 1.86 g/L and 1.39 g/L respectively. The bottle was covered with aluminum foil to avoid light penetrance.

3.3.1.4 Stock solution of vitamins

The stock solution of vitamins requires some cautions. The distilled water used to prepared vitamins stocks was autoclaved before being used. This precautionary measure is very important as it helps to keep the stock free of contamination. MS medium consists of following vitamins viz: nicotinic acid, pyridoxine HCl, thiamine HCl and myo inositol. The mass taken for each of individual vitamins is given table 3.2.

3.3.1.5 Stock solution of 1N NaOH

1N NaOH (Biomedical, germany) solution was prepared by dissolving 4 g of NaOH pellets in 100 ml of distilled water

3.3.1.6 Stock solution of Indole Acetic Acid (IAA)

100 mg (0.1 g) of IAA (Sigma Aldrich, USA) was weighted in electronic balance using aluminum foil to avoid sticking with paper. Ten ml of 1N NaOH was taken in volumetric cylinder and carefully poured in bottle. IAA was put in dissolvent and was gently stirred. After

Table 3.2 Stock solutions of MS Macro-nutrients, Micro-nutrients, Iron source and vitamins.

S.No.	Stock A* (g/L)		Stock B* (g/L)		Stock C* (g/L)		Stock D* (g/L)		Stock E* (g/L)		Stock F* (g/L)	
Chemical Required	NH_4NO_3	82.5	$MgSO_4.7H_2O$	18.5	KH_2PO_4	17	$MnSO_4$	1.69	FeNaEDTA	1.84	Myoinositol	10
	KNO_3	95					H_3BO_3	0.62	or		Nicotinicacid	0.050
	$CaCl_2$	22					KI	0.083	$FeSO_4.7H_2O$	1.39	Pyridoxine HCl	0.050
							$ZnSO_4.7H_2O$	0.86	NaEDTA	1.86		
							$NaMoO_4.5H_2O$	0.025			Thiamine HCl	0.010
							$CuSO_4.5H_2O$	0.0025				
							$CoCl_2.6H_2O$	0.0025				
Vol. of stock	1000 ml		1000 ml		1000 ml		1000 ml		1000 ml		1000 ml	
$L.^{-1}$ Vol.	20ml		20 ml		10ml		10ml		20ml		10ml	

*=Stock A, B & C = Macro-nutrients, Stock D = Micro-nutrients, Stock E= Iron Source and Stock F= Vitamins.

dissolving IAA 90 ml of distilled water was poured in bottle. The concentration of this stock solution is 1mg per ml. The bottle was tagged and stored in refrigerator at 4°C.

3.3.1.7 Stock solution of BAP

100 mg (0.1 g) of BAP (Sigma Aldrich, USA) was weighted in electronic balance using aluminum foil to avoid sticking with paper. Ten ml of 1N NaOH was taken in volumetric cylinder and carefully poured in bottle. BAP was put in dissolvent and was gently stirred. After dissolving BAP 90 ml of distilled water was poured in bottle. The concentration of this stock solution is 1mg per ml. The bottle was tagged and stored in refrigerator at 4°C.

3.4 Preparation of culture initiation medium

Culture initiation medium was prepared using all stock solutions of MS at their respective per liter volume. Sugar as carbon source was added at 30 g/L. The media was supplemented by BAP at 6 mg/L and IAA at 1 mg/L respectively. The media was solidified using Phyta gel (Phyto Technology USA) at 2 g/L.

3.5 Autoclaving of culture medium

Culture media was autoclaved at 121°C and 1.5 atm pressure for 15mins. The fungus and bacteria becomes inactive at temperature and pressure.

3.6 Growth room conditions

The 3000 lux light intensity was maintained in growth room with a photo period of 16 hours light period and 8 hours dark period and temperature was maintained at 27±2°C.

3.7 Culture initiation

The explants were cultured on initiation media as described earlier. Data for fungal contamination was recorded after 5-7 days of culture initiation. These explants were frequently transferred at 15 days interval to minimize browning. After 55-60 days the cultures were screened for endogenous bacterial contamination. The explants having 1-2 leaves (figure 3.1) and showing growth were selected and their leaves were aseptically excised in laminar flow hood. The excised leaves were put in polythene bag and PCR for virus indexing (BBTV) was performed. Only proliferated sucker free of any viral attack were subjected to optimization of plant growth regulator.

3.8 Molecular detection of BBTV *in vitro* culture of banana.

Leaf samples were taken from in vitro cultures of different Banana cultivars viz:, William-8818 hybrid Pisang and Brazilian. Field grown infected plants were taken as positive control. Total genomic DNA was extracted from these samples using modified CTAB method of DNA extraction. The extracted DNA was amplified using Rep B primer set (Rep B F 5'-CCAAATGGAGGAGAAGGAAAG-3'andRep B R 5'-GCCATAGACCCAAATTATTCTCCG-3') amplifying N component of BBTV (Banana Bunchy Top Virus). In addition B-actin gene from banana nuclear genome was also amplified using specific primer Actin F 5'-

ACTGTTCCTATATACGAAGG-3' and Actin R 5'-GAAAAGTGCTGAGCGAAG-3' as internal control.

The 20ul multiplex PCR reaction mixture contained about 25ng DNA template, Taq buffer (10mMTris-HCl, pH 8.8, 50mM KCl and 0.08% Nonidet P40) 1.5 mM $MgCl_2$, 200uM of each dNTPs, 1.5 units Taq DNA Polymerase (Thermo Scientific) and 25pmol/ul of each primer.

The thermal profile for multiplex PCR was initial denaturation at 96oC for 3 minutes followed by 35 cycles of denaturing at 96oC for 20 seconds, annealing at 52oC for 20 seconds and extension at 72oC for 40 seconds, and a final extension at 72oC for 20 minutes. 1Kb DNA ladder (Thermo Scientific) was used for comparison of bands in gel electrophoresis.

PCR products were run on 1% agarose gel at 100V for 35min using 1Kb DNA ladder as standard. After electrophoresis gel was visualized in Gel Documentation system. Rep B primer produced band of about 1000bp only from infected banana plant while bands of about 547bp with B-Actin gene specific primer were produced from both infected and healthy plants.

3.9 Treatments

Different combinations of IAA and BAP along with MS were prepared for optimizing multiplication protocols. These combinations are given in table 3.3, which includes four levels of BAP and three levels of IAA. The T0 treatment is control deprived of any plant growth hormones. In order to optimize rooting protocols, different combinations of auxins were used as described in table 3.4.

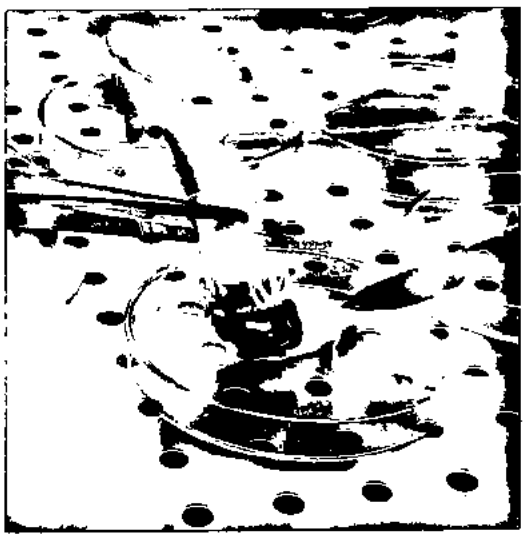


Figure 3.1 Proliferated suckers after 55-60 days

←Rep B (≈1000bp)
←Musa Actin
(≈547bp)

3.10 Collection of data

3.10.1 Contamination and survival percentages

Fungal contamination usually starts appearing as hyphael colonies grows after3-4 days of culture initiation. The presence of these colonies is visually distinguishable from their absence. Data for fungal contamination was recorded after one week of culture initiation by visual observation of fungal colonies in jars. Bacterial contamination was also visually calculated at regular intervals and survival percentage was finally calculated after eight weeks by counting the number of uncontaminated jars.

3.10.2 Mean number of shoots per explants (MSE)

Number of shoots per explants, ten plants per treatment, was calculated at 10 days intervals for period of 60 days and mean was calculated according to formula described

$$X = \frac{\sum xi}{N}$$

Where $\sum$= summation,

Xi=number of shoots and

n=number of observations

3.10.3 Longest shoot length (LSL)

The longest shoot length (cm), ten plants per treatment, was calculated after 60 days and means was calculated.

3.10.4 Fresh weight (FW)

Fresh weight (g) of, ten plants per treatment, was calculated after 60 days, according to following formula

$FW=W_1-W_2$

Where FW= Fresh weight in grams,

W_1=weight of culture vessel + proliferated explants and

W_2=weight of culture vessel

3.10.5 Number of leaves per shoot (LPS)

Individuals' shoots were transferred on rooting medium media and number of leaves per shoot was calculated after 45 days of transfer. Means was calculated for 10 plants per treatment.

3.10.6 Number of roots per shoot (RPS)

Individuals shoots were transferred on rooting medium media and number of roots per shoots was calculated after 45 days of transfer. Means was calculated for 10 plants per treatment.

3.10.7 Longest root length (LRL)

Longest root length (cm) was calculated after 45 days of transfer to rooting medium just before transferring to green house. Individual's shoots were taken out and adhering agar was washed away in running tap water. Ten plants per treatment were taken and mean was calculated.

3.11 Ex vitro hardening of plantlets

The detailed procedure for hardening of banana plants is given below

- Well developed plantlets having appropriate root length (≥5 cm) were removed from the culture vessels.

- The culture media was washed away from the roots with running tap water. This help to eradicate the chances for fungal or bacterial contamination as adhering media could be a source of contamination.
- These plantlets were transferred to opaque polythene bag (15x8 cm) filled with peat moss (STIRUP, Netherland) and kept in the green house (50% shade) for 20-30 days.
- The bags will be covered with transparent polythene sheet to maintain high humidity as tissue culture raise plants are produced under100 % humidity level and sudden decrease to this level might damage the plants. These polythene sheets were removed after 15 days of start of hardening process.
- After 35 days, survival percentages were calculated by counting the number of plantlets successfully hardened.

3.12 Statistical analysis

Analysis of variance (ANOVA) was performed in two way statistical analysis and interaction mean values for treatments over genotypes was calculated using software Statistix 8.1 version. The graphs were obtained using interaction mean values over treatments for each genotypes. LSD values at 5% level of significance were also computed.

Table 3.3: Different combinations of BAP and IAA.

Treatment No.	IAA (mg/Lt)	BAP (mg/Lt)
Treatment 0 (T0)	0	0
Treatment 1 (T1)	0.5	1
Treatment 2 (T2)	0.5	2.5
Treatment 3 (T3)	0.5	5
Treatment 4 (T4)	1	1
Treatment 5 (T5)	1	2.5
Treatment 6 (T6)	1	5.0

Table 3.4: Different combinations of IAA and NAA

Treatment No.	IAA (mg/Lt)	NAA (mg/Lt)
Treatment 0 (RT0)	0	0
Treatment 1 (RT1)	0.5	0
Treatment 2 (RT2)	0.5	1
Treatment 3 (RT3)	1	1
Treatment 4 (RT4)	0	0.5

Chapter 4

Results

Chapter 4

RESULTS

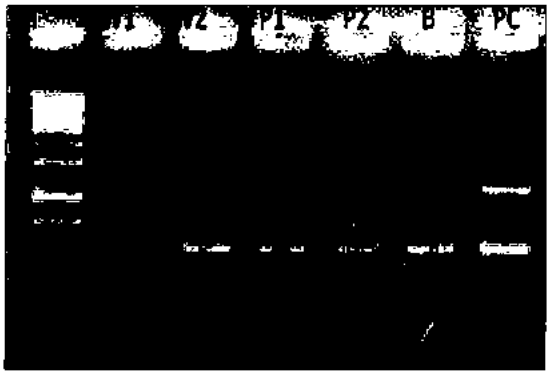
The data concerning different parameters for optimizing in vitro regenerations protocols were recorded, according to described procedure discussed in detail in chapter 3, and mean values were calculated followed by statistical analysis and results are presented in table 4.2-4.13. The original replicated data for following traits viz: mean number of shoots per explants (MSE), longest shoot length (LSL), fresh weight (FW), leaves per shoot (LPS), roots per shoot (RPS) and longest root length (LRL) are given in appendices while only percentage was calculated for contamination and survival. The findings of the study are briefly described below.

4.1 Contamination and survival percentages

The results for contamination and survival percentages are given in table 4.1. It was noticed that optimum Clorox concentration is very important for successful culture initiation. The Clorox at 50% concentration for 15 min give minimum contamination percentages, both fungal and bacterial, and optimum survival percentage.

4.2 PCR analysis of BBTV for different banana varieties

The results of PCR showed that the in vitro cultures of banana plant at their multiplication stage were free of BBTV as shown in figure 4.1.



Gel Doc Picture of multiplex PCR of Banana DNA samples with Rep B and Musa Actin Primers
M: 1Kb DNA ladder
LaneW1-W2: Banana DNA samples of Williams cultivar amplified with Rep B and Musa Actin Primers
Lane P1-P2: Banana DNA samples of pisang cultivar amplified with Rep B and Musa Actin Primers
Lane B: Banana DNA samples of Brazillian cultivar amplified with Rep B and Musa Actin Primers
PC: Positive Control

Figure 4.1 PCR analysis of BBTV for different banana varieties.

Table 4.1: Contamination and survival percentages for different Clorox concentrations

Clorox %	Exposure time (min)	Contamination %		Survival%
		Fungal %	Bacterial %	
0 %	5 min	90	10	Nil
	10 min	90	10	Nil
	15 min	80	20	Nil
10 %	5 min	80	90	10
	10 min	70	90	10
	15 min	70	90	10
25 %	5 min	50	70	30
	10 min	40	60	40
	15 min	30	60	40
50%	5 min	30	50	50
	10 min	30	30	70
	15 min	00	10	90

4.3 Mean number of shoots per explants (MSE)

The analysis of the data indicated that different combinations of cytokinin, benzyl amino purine (BAP) and auxin, indole acetic acid (IAA), had a highly significant effect on mean number of shoots per explants (MSE) of three varieties viz: Wiallium-8818 hybrid, Pisang and Brazilian, as represented in table 4.3.

The mean value for different combinations of plant growth regulators showed that highest value (6.53) for number of shoots per explants (MSE) was recorded in MS medium supplement with BAP and IAA at 2.5 and 1mg/l respectively (T5) which was statistically different from rest of treatments, followed by mean value of (5.54) shoots in the MS medium supplemented with BAP and IAA at 5.0 and 1mg/l respectively (T6) , while the minimum mean value (1.56 shoots) was observed in MS basal medium deprived of any plant growth regulators (T0) as shown in table 4.2 .

The means values recorded among the different varieties being tested showed that variety Wiallium-8818 hybrid gained maximum number of shoots per explants (4.94), followed by variety Brazillian (4.31) while least mean value (3.38) was recorded in variety Pisang. These values were statistically different from one another indicating presence of genotypic difference among all three varieties

The interaction of three varieties and different combinations of plant growth hormones showed significant difference for number of shoots per explants. The MS medium supplemented with BAP and IAA at 2.5 and 1 mg/l concentration (T5) showed highest

Table No. 4.2: Effect of various combinations of BAP and IAA on different Banana genotypes for mean number of shoots per explants.

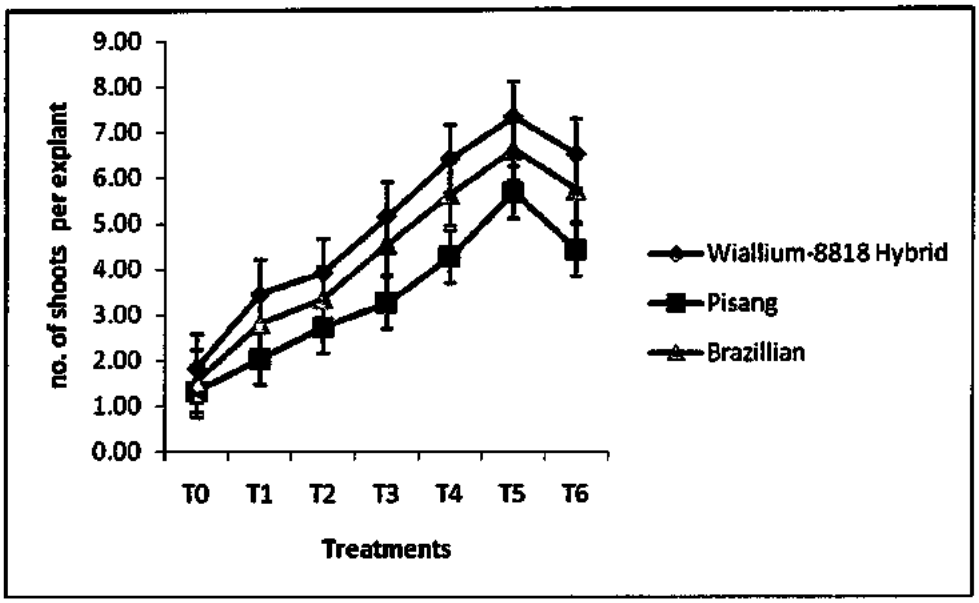
Treatments	BAP mg/L	IAA mg/L	Wiallium-8818 Hybrid	Pisang	Brazilian	Means
T0	0	0	1.82 IJ	1.31 K	1.54 JK	**1.56 F**
T1	1	0.5	3.44 G	2.03 I	2.80 H	**2.76 E**
T2	2.5	0.5	3.91F	2.71 H	3.35 G	**3.32 D**
T3	5	0.5	5.15 D	3.26 G	4.54 E	**4.32 C**
T4	1	1	6.41 B	4.27 E	5.62 C	**5.43 B**
T5	2.5	1	7.33 A	5.62 C	6.60 B	**6.53 A**
T6	5.0	1	6.50 B	4.41 E	5.70 C	**5.54 B**
Means			**4.94 A**	**3.38 C**	**4.31 B**	

Critical Value for Comparison of treatment: 0.1834
Critical Value for Comparison genotypes: 0.1200
Critical Value for Comparison interaction: 0.3176
Means followed by same letters are not significantly different using LSD test at 5 % level of probability.

Table No. 4.3: ANOVA of various combinations of BAP and IAA on mean number of shoot per explant of different Banana genotypes.

Source	DF	SS	MS	F calculated	P value
Replication	2	0.059	0.0297		
Treatments (T)	6	164.152	3587	738.70	0.0000
Varieties (V)	2	27.087	13.5434	365.68	0.0000
TxV	12	3.366	0.2805	7.57	0.0000
Error	40	1.481	0.0370		
Total	62	196.146			

Grand Mean 4.1965 CV 4.59



	T0	T1	T2	T3	T4	T5	T6
BAP mg/L	0	1	2.5	5	1	2.5	5
IAA mg/L	0	0.5	0.5	0.5	1	1	1

Figure 4.2: Mean values shoots per explant of different Banana genotypes affected by various BAP and IAA combinations

Figure 4.3 Cultures showing shoots per explant.

interaction mean value (7.33), followed by mean number of (6.50) shoots per explants value in MS medium supplemented with BAP and IAA at 5.0 and 1mg/l (T6) respectively for variety Wiallium-8818 hybrid. The variety Brazilian gained maximum number of shoots per explants (6.60) in medium supplemented with BAP and IAA at 2.5 and1 mg/l (T5) followed by mean value of 5.70 shoots per explants in MS medium supplemented by BAP and IAA at 5.0 and 1mg/l concentration (T6). However, interaction means values of treatments and the variety Pisang showed that highest mean value (5.62) in MS medium supplemented with BAP and IAA at 2.5 and 1 mg/l (T5) followed by mean value of (4.41) shoots per explants in MS medium supplemented with BAP and IAA at 5.0 and 1mg/l concentration (T6). The least mean interaction values for Wiallium-8818 hybrid (1.82), Pisang (1.31) and Brazilian (1.54) was recorded in MS medium deprived of any plant growth hormones (T0) . These least values in control indicated the significance of auxin and cytokinin in optimizing regeneration protocol.

4.4 Longest shoot length (LSL)

The analysis of the data indicated that different combinations of cytokinin, benzyl amino purine (BAP) and auxin, indole acetic acid (IAA), had a highly significant effect on longest shoot length (cm) of three varieties viz: Wiallium-8818 hybrid, Pisang and Brazilian,as represented in table .4.5.

The mean value for different combinations of plant growth regulators showed that highest value (8.95cm) was recorded in MS medium supplement with BAP and IAA at 1 and 1mg/l respectively (T4) which was statistically different from rest of treatments, followed (7.69cm) in the MS medium supplemented with BAP and IAA at 5 and 0.5mg/l respectively (T3) , while the least value (3.07cm) was obtained in MS basal medium deprived of any plant growth regulators

Table 4.4 : Effect of various combinations of BAP and IAA on longest shoot length(cm) of different Banana genotypes.

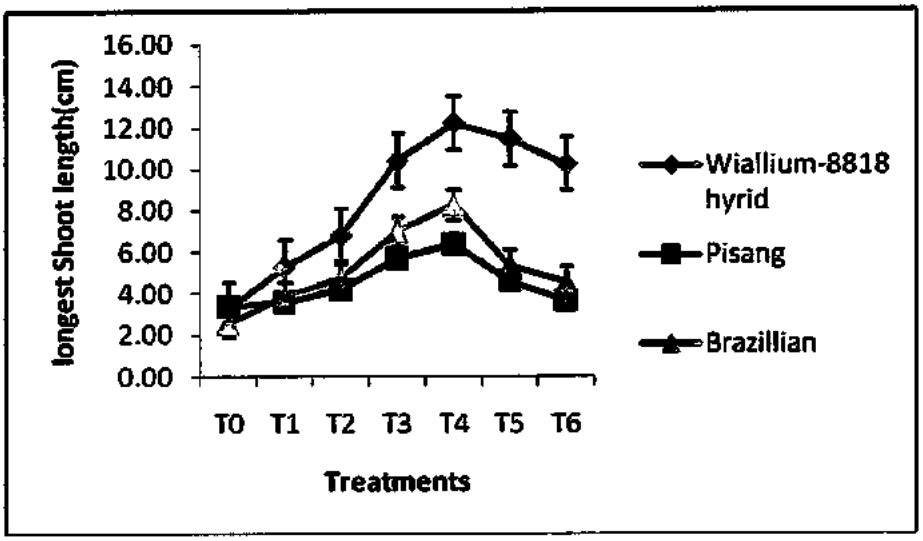
Treatments	BAP mg/L	IAA mg/L	Wiallium-8818 hybrid	Pisang	Brazilian	Mean
T0	0	0	3.26 M	3.38 LM	2.57 N	**3.07 G**
T1	1	0.5	5.31 H	3.59 KL	3.84 K	**4.25 F**
T2	2.5	0.5	6.82 E	4.21 J	4.72 I	**5.25 E**
T3	5	0.5	10.39 C	5.69 G	6.99 E	**7.69 B**
T4	1	1	12.20 A	6.37 F	8.27 D	**8.95 A**
T5	2.5	1	11.44 B	4.61 I	5.36 H	**7.14 C**
T6	5.0	1	10.23 C	3.65 KL	4.55 I	**6.14 D**
Means			**8.52 A**	**4.50 C**	**5.18 B**	

Alpha = 0.05
Critical Value for Comparison for treatment mean = 0.1719
Critical Value for Comparison for genotype mean = 0.1126
Critical Value for Comparison for interactions = 0.2978
Means followed by same letters are not significantly different using LSD test at 5 % level of probability.

Table No 4.5: ANOVA of various combinations of BAP and IAA on longest shoot length(cm) of different Banana genotypes.

Source	DF	SS	MS	F calculated	P value
Replication	2	0.058	0.0288		
Treatments (T)	6	225.508	37.5847	1154.06	0.0000
Varieties (V)	2	194.424	97.2120	2984.95	0.0000
TxV	12	72.474	6.0395	185.45	0.0000
Error	40	1.303	0.0326		
Total	62	493.766			

Grand Mean = 6.0690
Coefficient of Variation (CV) = 2.97



	T0	T1	T2	T3	T4	T5	T6
BAP mg/L	0	1	2.5	5	1	2.5	5
IAA mg/L	0	0.5	0.5	0.5	1	1	1

Figure 4.4: Longest shoot length of different Banana genotypes affected by various BAP and IAA combinations

Figure 4.5: Culture showing longest shoot length (cm).

(T0). The means values recorded among the different varieties being tested showed that variety Wiallium-8818 hybrid gained maximum shoot length (8.52cm), followed by variety Brazillian (5.18cm) while least mean value (4.50cm) was recorded in variety Pisang. These values were statistically different from one another indicating presence of genotypic difference among varieties.

The interaction of three varieties and different combinations of plant growth hormones showed significant difference for longest shoot length. The MS medium supplemented with BAP and IAA at 1 and1 mg/l concentration (T4) showed highest interaction mean value (12.20cm), followed by shoot length value (11.44cm) in MS medium supplemented with BAP and IAA at 2.5 and1mg/l (T5) for variety Wiallium-8818 hybrid. The variety Brazilian gained maximum shoot length (8.27cm) in medium supplemented with BAP and IAA at 1 and1mg/l (T4) followed by shoot length of (6.99cm) in medium supplemented by BAP and IAA at 5 and 0.5mg/l concentration (T3). However, interaction means values of treatments and varieties showed that variety Pisang gained highest mean value (6.37cm) in medium supplemented with BAP and IAA at 1 and 1mg/l (T4) followed by mean shoot length (5.69cm) in MS medium supplemented with BAP and IAA at 5 and 0.5 mg/l concentration (T3). The least mean interaction values for Wiallium-8818 hybrid (3.26cm), Pisang (3.38cm) and Brazilian (2.57cm) was recorded in MS basal medium deprived of any plant growth hormones (T0). These least value in control indicates significance of plant growth hormones on aforementioned trait.

4.5 Fresh weight (FW)

The analysis of the data indicated that different combinations of cytokinin, benzyl amino purine (BAP) and auxin, indole acetic acid (IAA), had a highly significant effect on fresh weight (FW) of three varieties viz: Wiallium-8818 hybrid, Pisang and Brazilian, as represented in table 4.7

Table 4.6: Effect of various combinations of BAP and IAA on fresh weight (g)of different Banana genotypes.

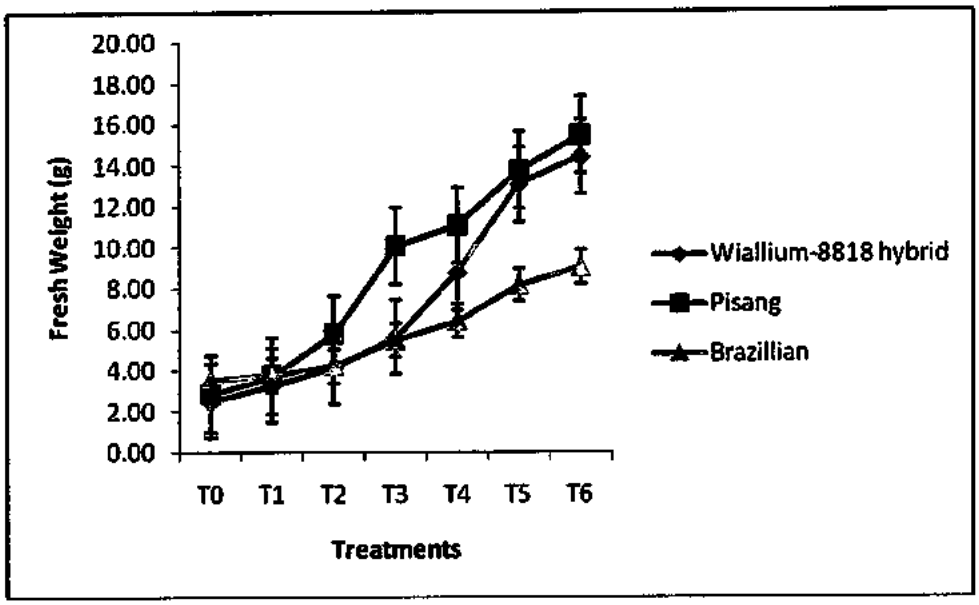
Treatments	BAP mg/L	IAA mg/L	Wiallium-8818 hybrid	Pisang	Brazilian	Mean
T0	0	0	2.49 Q	2.82 P	3.53 N	**2.95 G**
T1	1	0.5	3.27 O	3.72 N	3.76 N	**3.58 F**
T2	2.5	0.5	4.16 M	5.78 K	4.20 M	**4.71 E**
T3	5	0.5	5.61 KL	10.06 F	5.48 L	**7.05 D**
T4	1	1	8.77 H	11.06 E	6.40 J	**8.74 C**
T5	2.5	1	13.08 D	13.74 C	8.17 I	**11.66 B**
T6	5.0	1	14.39 B	15.45 A	9.05 G	**12.96 A**
Means			**7.40 B**	**8.95 A**	**5.80 C**	

Alpha = 0.05
Critical Value for Comparison for treatment mean = 0.1500
Critical Value for Comparison for replication mean = 0.0982
Critical Value for Comparison for interactions means = 0.2598
Means followed by same letters are not significantly different using LSD test at 5 % level of probability.

Table No 4.7: ANOVA of various combinations of BAP and IAA on mean fresh weight(g) of different Banana genotypes.

Source	DF	SS	MS	F calculated	P value
Replication	2	0.20	0.1		
Treatments (T)	6	833.16	138.860	5604.29	0.0000
Varieties (V)	2	104.52	52.261	2109.23	0.0000
TxV	12	102.36	8.530	344.26	0.0000
Error	40	0.99	0.025		
Total	62	1041.24			

Grand Mean = 7.3820
Coefficient of Variation (CV) = 2.13



	T0	T1	T2	T3	T4	T5	T6
BAP mg/L	0	1	2.5	5	1	2.5	5
IAA mg/L	0	0.5	0.5	0.5	1	1	1

Figure 4.6: Fresh weight of different Banana genotypes affected by various BAP and IAA combinations

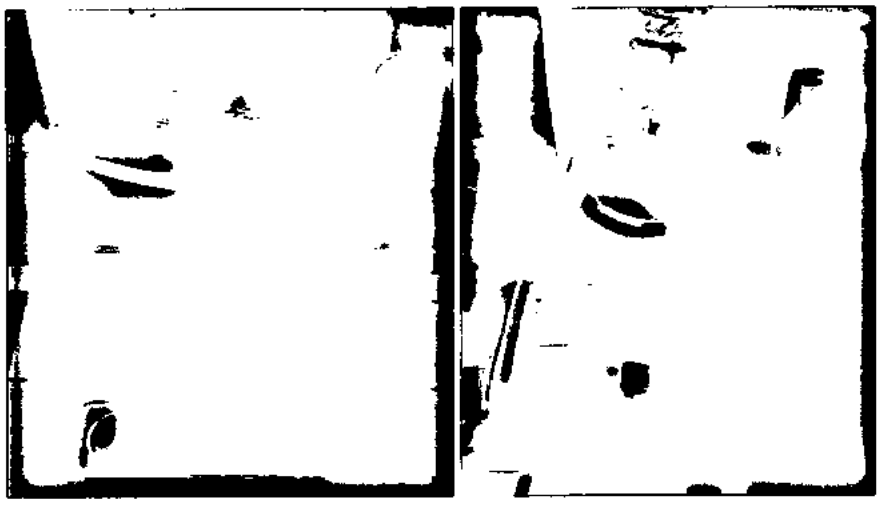


Figure 4.7: Culture showing fresh weight (g).

The highest fresh weight mean value (12.96 g) for different combinations of plant growth regulators was recorded in MS medium supplement with BAP and IAA at 5 and 1mg/l respectively (T6) which was statistically different from rest of treatments, followed by mean fresh weight value (11.66 g) in the MS medium supplemented with BAP and IAA at 2.5 and 1mg/l respectively (T5), while the least mean fresh weight value (2.95 g) was observed in MS basal medium deprived of any plant growth regulators (T0).

The means values recorded among the different varieties being tested showed that variety Pisang gained maximum fresh weight (8.95g) followed by mean fresh weight of 7.40 g for variety Wiallium-8818 hybrid while least mean value (5.80 g) was recorded in variety Brazillian. These values were statistically different from one another indicating presence of genotypic difference among varieties.

The interaction among varieties and different combinations of plant growth hormones showed significant difference for fresh weight. The MS medium supplemented with BAP and IAA at 5 and 1mg/l concentration (T6) showed highest fresh weight interaction mean value (15.45 g), followed by mean fresh weight value of 13.74 g in MS medium supplemented with BAP and IAA at 2.5 and 1mg/l (T5) for variety Pisang. The variety Wiallium-8818 hybrid gained highest fresh weight (14.39 g) in medium supplemented with BAP and IAA at mg/l (T6) followed by mean fresh weight value of 13.08 g in medium supplemented by BAP and IAA at 2.5 and 1 mg/l concentration (T5). However, interaction means values among treatments and varieties showed that variety Brazilian gained highest mean fresh weight value (9.05 g) in medium supplemented with BAP and IAA at 5 and 1mg/l (T6) followed by mean fresh weight value of 8.17 g in MS medium supplemented with BAP and IAA at 2.5 and 1 mg/l concentration

(T5). The least fresh weight mean interaction values for Wiallium-8818 hybrid (2.49 g), Pisang (2.82 g) and Brazilian (3.53 g) was recorded in MS medium deprived of any plant growth hormones (T0). These highly significant least values in Treatment 0 (T0) indicates the significance of combination of auxin and cytokinin in optimizing regeneration protocol.

4.6 Leaves per shoot (LPS)

The analysis of the data indicated that different combinations of auxins, indole acetic acid (IAA) and naphthalene acetic acid (NAA) had a highly significant effect on leaves per shoot (LPS) of three varieties viz: Wiallium-8818 hybrid, Pisang and Brazilian,as shown in table 4.9

The highest mean leaves per shoot value (5.70) for different combinations of plant growth regulators was recorded in MS medium supplement with NAA at 0.5 mg/l (RT4) which was statistically different from rest of treatments, followed by mean leaves per shoot value (5.00) in the MS medium supplemented with NAA and IAA each at 1 mg/l respectively (RT3), while the least mean leaves per shoot value (2.56) was observed in MS basal medium deprived of any plant growth regulators (RT0) as shown in table 4.8

The means values recorded among the different varieties being tested showed that variety Wiallium-8818 hybrid gained maximum value (4.47) followed by mean leaves per shoot value (4.08) for variety Pisang while least mean value (3.83) was recorded in variety Brazilian. These values were statistically different from one another indicating presence of genotypic difference among varieties.

The interaction among varieties and different combinations of plant growth hormones showed significant difference for mean leaves per shoot. The MS medium supplemented with

Table 4.8 : Effect of various combinations of IAA and NAA on leaves per shoot of different Banana genotypes.

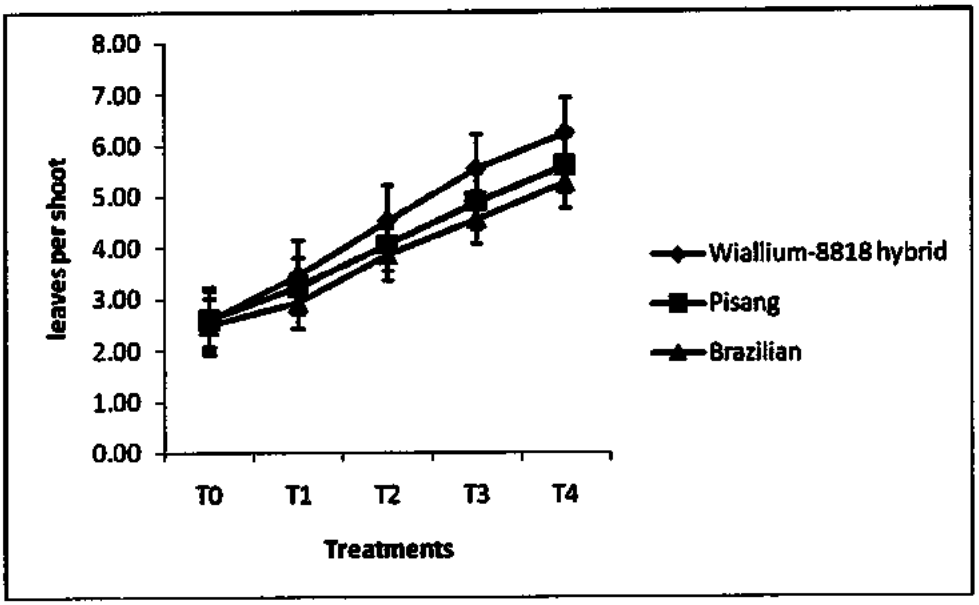
Treatment	IAA mg/L	NAA mg/L	Wiallium-8818 hybrid	Pisang	Brazilian	Mean
RT0	0	0	2.57 HI	2.60 HI	2.50 I	**2.56 E**
RT1	0.5	0	3.47 F	3.23 FG	2.93 GH	**3.21 D**
RT2	0.5	1	4.53 D	4.07 E	3.87 E	**4.16 C**
RT3	1	1	5.53 B	4.90 CD	4.57 D	**5.00 B**
RT4	0	0.5	6.23 A	5.60 B	5.27 BC	**5.70 A**
Mean			**4.47 A**	**4.08 B**	**3.83 C**	

Alpha = 0.05
Critical Value for Comparison for treatment mean = 0.221819
Critical Value for Comparison for varietal mean = 0.1718
Critical Value for Comparison for interactions means = 0.3841
Means followed by same letters are not significantly different using LSD test at 5 % level of probability.

Table No 4.9 : ANOVA of various combinations of IAA and NAA on mean leaves per shoot of different Banana genotypes.

Source	DF	SS	MS	F calculated	P value
Replication	2	0.1764	0.0882		
Treatments (T)	4	58.9098	14.7274	279.21	0.0000
Varieties (V)	2	3.1164	1.5582	29.54	0.0000
TxV	8	0.9236	0.1154	2.19	0.0599
Error	28	1.4769	0.0527		
Total	44	64.6031			

Grand Mean = 4.1244
Coefficient of Variation (CV) = 5.57



	T0	T1	T2	T3	T4
IAA mg/L	0	0.5	0.5	1	0
NAA mg/L	0	0	1	1	0.5

Figure 4.8: Leaves per shoot of different Banana genotypes affected by various NAA and IAA combinations

Figure 4.9: Cultures showing Leaves per shoot.

NAA at 0.5 mg/l concentration (RT4) showed maximum leaves per shoot interaction mean value (6.23), followed by mean leaves per shoot value (5.53) in MS medium supplemented with IAA and NAA each at 1 mg/l (RT3) for variety Wiallium-8818 hybrid. The variety Pisang gained maximum mean leaves per shoot value (5.60) in medium supplemented with NAA at 0.5 mg/l (RT4) followed by mean leaves per shoot value (4.90) in medium supplemented by NAA and IAA each at 1 mg/l concentration (RT3). However, interaction means values among treatments and varieties showed that variety Brazilian gained maximum mean leaves per shoot value (5.27) in medium supplemented with NAA at 0.5 mg/l (RT4) followed by mean leaves per shoot value (4.57) in MS medium supplemented with NAA and IAA each at 1 mg/l concentration (RT3). The least leaves per shoot mean interaction values for Wiallium-8818 hybrid (2.57), Pisang (2.60) and Brazilian (2.50) was recorded in MS medium deprived of any plant growth hormones (RT0). These highly significant least values in control indicate the significant contribution of auxins in optimizing regeneration protocol.

4.7 Roots per shoots (RPS)

The analysis of the data indicated that different combinations of auxins, indole acetic acid (IAA) and napthalenne acetic acid (NAA) had a highly significant effect on roots per shoots (RPS) of three varieties viz: Wiallium-8818 hybrid, Pisang and Brazilian as shown in table 4.11

The highest mean roots per shoots value (4.67 roots) for different combinations of plant growth regulators was recorded in MS medium supplement with NAA and IAA at 1mg/l respectively (RT3) which was statistically different from rest of treatments, followed by mean

Table 4 .10 : Effect of various combinations of IAA and NAA on roots per shoot of different Banana genotypes.

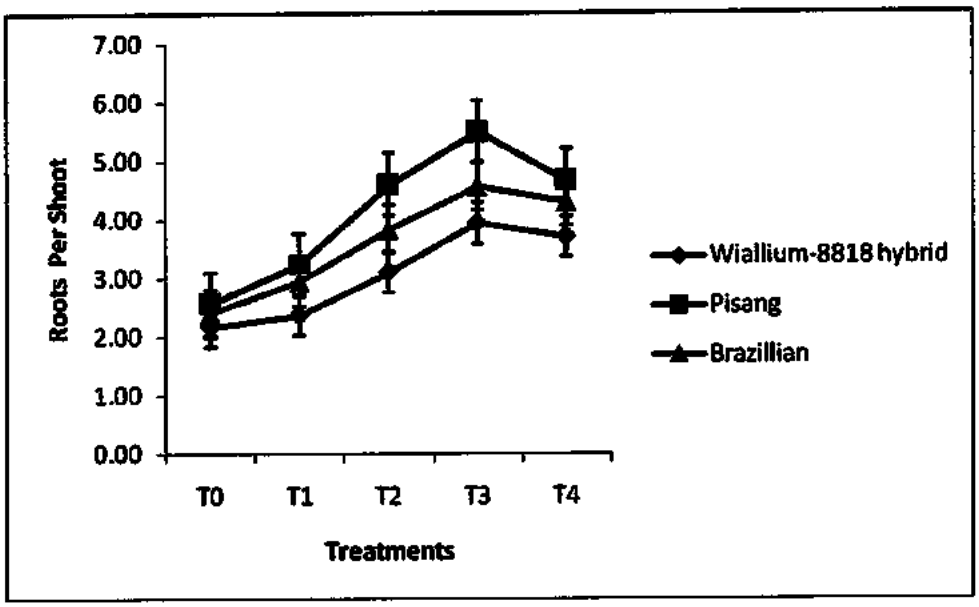
Treatment	IAA mg/L	NAA mg/L	Wiallium-8818 hybrid	Pisang	Brazilian	Mean
RT0	0	0	2.17 G	2.57 FG	2.40 G	**2.38 E**
RT1	0.5	0	2.37 G	3.23 E	2.93 EF	**2.84 D**
RT2	0.5	1	3.10 E	4.60 B	3.83 D	**3.84 C**
RT3	1	1	3.93 CD	5.50 A	4.57 B	**4.67 A**
RT4	0	0.5	3.70 D	4.67 B	4.30 BC	**4.22 B**
Mean			**3.05 C**	**4.11 B**	**3.61 B**	

Standard Error for Comparison treatments 0.1133
Standard Error for Comparison Replication 0.0877
Standard Error for Comparison interaction 0.1962
Means followed by same letters are not significantly different using LSD test at 5 % level of probability.

Table No 4.11: ANOVA of various combinations of IAA and NAA on mean number of roots per shoot of different Banana genotypes.

Source	DF	SS	MS	F calculated	P value
Replication	2	0.0031	0.00156		
Treatments (T)	4	32.8409	8.21022	142.18	0.0000
Varieties (V)	2	8.4324	4.21622	73.01	0.0000
TxV	8	1.5031	0.18789	3.25	0.0095
Error	28	1.6169	0.05775		
Total	44	44.3964			

Grand Mean 3.5911 CV 6.69



	T0	T1	T2	T3	T4
IAA mg/L	0	0.5	0.5	1	0
NAA mg/L	0	0	1	1	0.5

Figure 4.10: Roots per shoot of different Banana genotypes affected by various NAA and IAA combinations

Figure 4.11: Culture showing Roots per shoot

roots per shoots value (4.22) in the MS medium supplemented with NAA at 0.5 mg/l respectively (RT4), while the least mean roots per shoots value (2.38 roots) was observed in MS basal medium deprived of any plant growth regulators (RT0) as shown in table 4.10

The means values recorded among the different varieties being tested showed that variety Pisang gained maximum roots (4.11 roots) followed by mean roots per shoot value of 3.61 roots for variety Brazilian while least mean roots per shoot value (3.05 roots) was recorded in variety Wiallium-8818 hybrid. These values were statistically different from one another indicating presence of genotypic difference among varieties as shown in table 4.10

The interaction among varieties and different combinations of plant growth hormones (Auxins) showed significant difference for roots per shoot. The MS medium supplemented with NAA and IAA at 1mg/l each concentration (RT3) showed maximum roots per shoot interaction mean value (5.50 roots), followed by mean roots per shoot value of 4.67 roots in MS medium supplemented with NAA at 0.5 mg/l (RT4) for variety Pisang. The variety Brazilian gained maximum roots per shoot (4.57 roots) in medium supplemented with NAA and IAA at 1mg/l each (RT3) followed by mean roots per shoot value of 4.30 roots in medium supplemented by NAA at 0.5mg/l concentration (RT4). However, interaction means values among treatments and varieties showed that variety Wiallium-8818 hybrid gained maximum mean roots per shoot value (3.93 roots) in medium supplemented with NAA and IAA at 1mg/l each (RT3) followed by mean roots per shoot value of 3.70 roots in MS medium supplemented with NAA at 0.5 mg/l concentration (RT4). The least roots per shoot mean interaction values for Wiallium-8818 hybrid (2.17 roots), Pisang (2.57 roots) and Brazilian (2.40 roots) was recorded in MS medium deprived

of any plant growth hormones (RT0) as shown in table 4.10. These highly significant least values (RT0) indicate the significant contribution of IAA and NAA in optimizing roots induction.

4.8 Longest root length (LRL)

The analysis of the data indicated that different combinations of auxins, indole acetic acid (IAA) and napthalenne acetic acid (NAA) had a highly significant effect on roots length of three varieties viz: Wiallium-8818 hybrid, Pisang and Brazilian as represented in table 4.13

The highest mean roots length value (5.58 cm) for different combinations of plant growth regulators was recorded in MS medium supplement with NAA and IAA at 1 and 0.5 mg/l respectively (RT2) which was statistically different from rest of treatments except RT3, followed by mean roots length value (5.46 cm) in the MS medium supplemented with NAA and IAA at 1mg/l each (RT3), while the least mean roots length value (4.11 cm) was observed in MS basal medium deprived of any plant growth regulators (RT0) as shown in table 4.12.

The means values recorded among the different varieties being tested showed that variety Pisang gained maximum root length (5.48 cm) followed by mean root length value of 5.09 cm for variety Brazilian while least mean roots length value (4.40 cm) was recorded in variety Wiallium-8818 hybrid. These values were statistically different from one another indicating presence of genotypic difference among varieties as shown in table 4.12.

The interaction among varieties and different combinations of plant growth hormones (Auxins) showed significant difference for roots length. The MS medium supplemented with NAA and IAA at 1 and 0.5mg/l concentration respectively (RT2) showed

Table 4.12 : Effect of various combinations of IAA and NAA on longest root length (cm)of different Banana genotypes.

Treatment	IAA mg/L	NAA mg/L	Wiallium-8818 hybrid	Pisang	Brazilian	Mean
RT0	0	0	4.15 GH	4.28 G	3.89 H	**4.11 D**
RT1	0.5	0	4.49 FG	5.37 C	5.18 CD	**5.01 B**
RT2	0.5	1	4.74 EF	6.25 A	5.7550 B	**5.58 A**
RT3	1	1	4.38 G	6.24 A	5.7620 B	**5.46 A**
RT4	0	0.5	4.23 GH	5.28 C	4.87 DE	**4.79 C**
Mean			**4.40 C**	**5.48 A**	**5.09 B**	

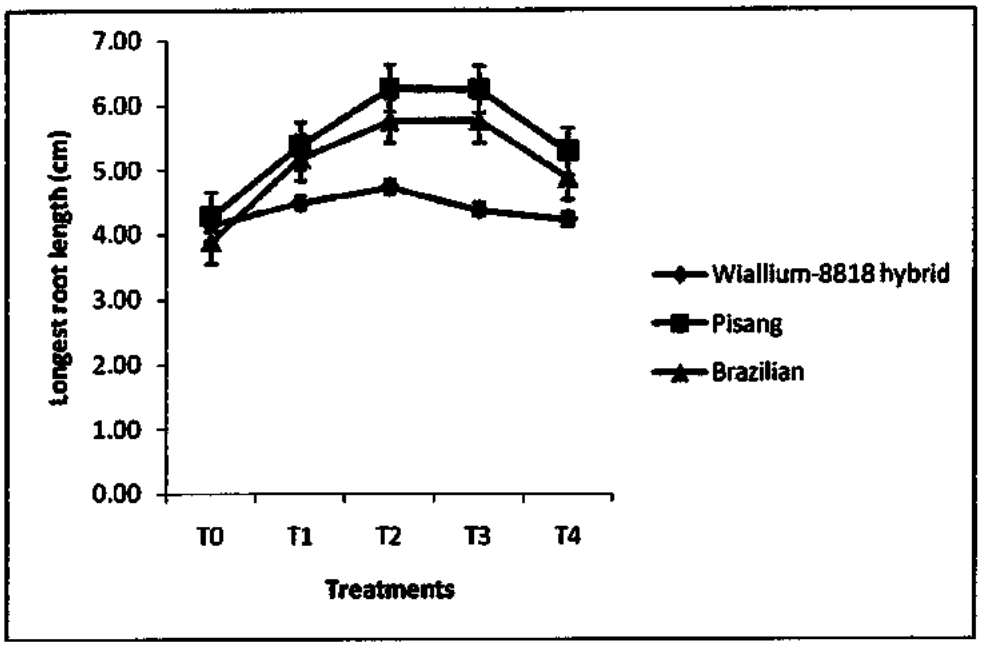
Critical

Value for Comparison treatment 0.1968
Critical Value for Comparison varietal mean 0.1524
Critical Value for Comparison interaction 0.3408
Means followed by same letters are not significantly different using LSD test at 5 % level of probability.

Table No 4.13: ANOVA of various combinations of IAA and NAA on longest root length (cm)of different Banana genotypes.

Source	DF	SS	MS	F calculated	P value
Replication	2	0.1114	0.05572		
Treatments (T)	4	12.4991	3.12477	75.24	0.0000
Varieties (V)	2	9.0452	4.52261	108.90	0.0000
TxV	8	3.3027	0.41284	9.94	0.0000
Error	28	1.1628	0.04153		
Total	44	26.1213			

Grand Mean 4.9910 CV 4.08



	T0	T1	T2	T3	T4
IAA mg/L	0	0.5	0.5	1	0
NAA mg/L	0	0	1	1	0.5

Fig 4.12: longest Root length of different Banana genotypes affected by various NAA and IAA combinations

Figure 4.13: Culture showing longest Root length.

maximum roots length interaction mean value (6.25 cm), followed by mean roots length value of 6.24 cm in MS medium supplemented with NAA and IAA at 1 mg/l each (RT3) for variety Pisang. The variety Brazilian gained maximum roots length (5.7620cm) in medium supplemented with NAA and IAA at 1mg/l each (RT3) followed by mean roots length value of 5.7550 cm in medium supplemented by NAA and IAA at 1mg/l and 0.5 mg/l concentration (RT2) which was statistically similar to value recorded for RT3. However, interaction means values among treatments and varieties showed that variety Wiallium-8818 hybrid gained maximum mean roots length value (4.74 cm) in medium supplemented with NAA and IAA at 1 and 0.5 mg/l (RT2) followed by mean roots length value of 4.49 cm in MS medium supplemented with IAA at 0.5mg/l concentration (RT1). The least roots length mean interaction values for Wiallium-8818 hybrid (4.15 cm), Pisang (4.28 cm) and Brazilian (3.89 cm) was recorded in MS medium deprived of any plant growth hormones (RT0) as shown in table 4.12. These highly significant least values in (RT0) indicates the significant contribution of IAA and NAA in optimizing roots induction protocol.

4.9 Ex vitro hardening of plantlets

Our experiment showed that peat moss can be successfully used at commercial scale for successful hardening of banana plants with over 99% survival could be easily achieved by adopting the necessary precaution during hardening process.

Figure 4.14 Successful primary hardened banana plants

Chapter 5

Discussion

Chapter 5

DISCUSSION

The hormone BAP is well known plant growth cytokinin which promotes the growth of shoot and auxiliary buds. This particular hormone is widely used in plant tissue culture experiments to boost multiplication stages. The IAA is a well known auxin which promotes the growth of roots. Besides root development, IAA is also used widely in multiplication protocols along with cytokinins.

The present study was conducted using four levels of BAP (0, 1. 2.5 and 5.0 mg/L) and three levels of IAA (0, 0.5 and 1.0 mg/L) MS medium. These two hormones were employed using seven treatments viz: T0-T6 and mean values of different morphological traits viz: mean number of shoots, longest shoot length and fresh weight were calculated for each treatment.

In present study, maximum number of shoots, longest shoot length and fresh weight were increased as level of BAP (0-5 mg/L) and IAA (0-1.0 mg/L) was simultaneously increased in applied treatments. Maximum number of shoots per explants (7.33) for variety Wiallium-8818 hybrid was obtained in MS medium supplement with BAP at 2.5 mg/L and IAA at 1 mg/L while at control mean value (1.82) for same aforementioned trait of same variety was comparatively low (75.17 %). Similar kind of results was obtained in an experiment to optimize best combination of different combinations of plant growth hormones (BAP, IAA and Kinetin). The combination of BAP and IAA at 2.0 mg/L and 0.5 mg/L along with MS gave highest number of shoots buds (7.85±0.26) for *Musa* species (Anbazhagan *et al*, 2014). Different kinds of results were reported by many other scientists as in a optimization study, maximum number of shoots (9.61) was found slightly higher (31.10 %) as compared to our results, in MS medium with BAP

at 0.56 mg/L (2.5 µM) concentration for Tantuk banana genotype (Elhory *et al.*, 2009). Similarly in another study for optimizing invitro protocol for three banana exotic genotypes (GCTCV-215,Yangambi Km-5, FHIA-23), it was found that maximum number of shoots per explants value (8.75 shoots) was obtained in MS medium supplemented with BAP and IAA at 4 mg/L and 0.5 mg/L respectively for GCTCV-215 banana genotypes (Qamar *et al*, 2015). These different responses to aforementioned trait might be of variable genotypic responses to different levels of BAP.

We found that length of shoots was increased as level of BAP and IAA was simultaneously increased up to 1 mg/L concentration and maximum shoot length (12.20 cm) was obtained in MS medium supplemented with BAP and IAA each at 1 mg/L concentration. Similar kind of results were obtained in optimization study to explore the best combination of different combinations of plant growth hormones (BAP and Kinetin), found that combination of BAP and Kinetin at 0.5 mg/L and 1.0 mg/L respectively, along with MS gave highest length of shoots buds (8.9±1.142cm) for different *Musa* species (Miilon *et al.*, 2013). This showed the significance of using BAP along with Kinetin in optimizing banana invitro micropropagation protocols. Contrary to our findings, a experiment to optimize regeneration protocol for banana cultivar *Meitei hei*, it was found that highest mean value for maximum shoot length (6.25cm) was obtained in full strength MS medium supplemented with BAP at 0.5 mg/Land NAA at 1.0 mg/L concentration (Lalrinsanga *et al.*, 2013). This dissimilarity from our findings reflects presence of some genotypic differences or might be of reduced response of NAA as compared to IAA in our study of banana micropropagation.

In present study we found that highest mean values (12.96g) for fresh weight was obtained in MS medium supplemented with BAP and IAA at 5.0 mg/Land 1 mg/L respectively.

These values were different as obtained from other studies. A study conducted to determine effect of BAP on *Musa acuminata* cv. Berangan, revealed that highest mean value (4.04±0.45 g) for fresh mass after 30 days was obtained in MS medium supplemented with BAP at 7.43 mg/L(33 µM) (Jafari *et al.,* 2010). Similarly another study conducted on banana cultivar GCTCV-215, gained highest mean fresh value (8.77 g) after 45 days on MS medium supplemented with BAP and IAA at 4.0 mg/Land 0.5 mg/L respectively (Qamar *et al.,* 2015). On comparative analysis of results obtained by previous and present study revealed that mean fresh weight is directly proportional to number of days for recording aforesaid trait.

In the present study it was found that highest mean values (5.7 leaves) for maximum number of leaves per shoot was obtained in MS medium supplemented with NAA at 0.5 mg/L. The below mentioned previous studies showed that different combinations of plant growth regulators (auxins / cytokines / auxins + cytokines) were applied by different researchers to evaluate number of leaves per shoot. Our results are in accordance with an experimental study using auxins only in optimizing micropropagation protocol of Grand Naine banana genotype gained mean number of leaves per shoots (5.66 leaves) in half strength MS supplemented with IBA and NAA each at 1 mg/L concentration (Ahmed *et al.*, 2014). Contrary to present results, a study conducted to determine affect of BAP on *Musa sp.* cv. Banana BARI-1 revealed highest mean value for leaves per shoots (7.00 leaves) was obtained in MS medium supplemented with BAP and NAA at 7.5 mg/L and 0.5 mg/L respectively after thirty days (Al-amin *et al.,* 2009). This difference might be of using combination of cytokinins (BAP) along with the auxins (NAA) contrary to our treatments of using only auxins (NAA).

In the present study maximum number of roots per shoot (5.50) was obtained in MS medium supplemented with IAA and NAA each at 1 mg/L concentration. Our result is partially

similar with a study conducted on optimization of root induction protocol for banana cv. Grand Naine and highest number of roots per shoot (6.4) using IAA at 1.0 mg/L concentration (Miilion *et al.*, 2013). This similarity is showing a positive contribution of IAA in increasing number of roots per shoot.

In our study maximum root length (6.25 cm) was obtained in MS medium with IAA at 0.5 mg/L concentration. Similar root length (7.80 cm) for banana Grand Naine genotype was obtained in another study by using half strength MS along with activated charcoal (200 mg/L) and IBA (1.0 mg/L) concentration (Ahmed *et al.*, 2014). This showed that root length is depended on number of factors including auxins (type and concentration), MS strength (full / half), concentration of activated charcoal and genotype. These similarities in previous and present studies also suggest that auxins (IBA and IAA) at 1 mg/L concentration might be useful in obtaining long roots for different banana genotypes.

CONCLUSION

Pakistan is spending huge amount of money on import of banana fruit, from its neighbors countries mainly from India, to meet the country's domestic demand. Over the last decade, India has emerged as one of the largest banana exporter among Asian countries. In early 90's, almost 99% of banana cultivation area was severely hit by a viral attack now called as Banana Bunchy Top Virus (BBTV). This virus destroyed the banana fields completely and still persists in major banana growing areas of Sindh area but in less active form. The yield of banana crop grown in our country is comparatively low as compared to major banana producing countries like India. There are several reasons behind this low yield which includes the non availability of disease free planting material and lack of awareness about exotic genotypes. This study was conducted to address these issues through economical use of plant tissue technology for efficient protocol optimization for various exotic banana genotypes. This study has demonstrated use of various plant growth regulators for optimizing tissue culture protocol for fast multiplication of various banana genotypes. Benzyl amino purine (BAP) is found to induce multiple shoots along with Indole Accetic Acid (IAA). Therefore it is concluded that BAP could be employed for regeneration protocol.

In the future prospective of using these exotic varieties, exploration and incorporation of biotechnological advancements, like genetic engineering for BBTV and other viral attack resistance, could be used to address aforementioned low yield of banana crop to manufacture high yielding banana resistant genotypes which would be helpful in producing low cost banana fruits.

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Appendix 1: Mean values of different banana genotypes for mean number of shoots per explant

Varieties	Treatments	Replication 1	Replication 2	Replication 3	Mean	Standard deviation
Wiallium-8818 hybrid	T0	1.98	1.59	1.89	1.82	±0.204206
	T1	3.47	3.43	3.43	3.44	±0.023094
	T2	3.93	3.85	3.95	3.91	±0.052915
	T3	5.15	5.18	5.12	5.15	±0.03
	T4	6.43	6.42	6.37	6.41	±0.032146
	T5	7.76	7.45	6.78	7.33	±0.500899
	T6	6.45	6.27	6.79	6.5	±0.264071
Pisang	T0	1.28	1.32	1.32	1.31	±0.023094
	T1	2.1	2	1.98	2.03	±0.064291
	T2	2.75	2.5	2.89	2.71	±0.197569
	T3	3.27	3.28	3.22	3.26	±0.032146
	T4	4.26	4.3	4.25	4.27	±0.026458
	T5	5.67	5.28	5.32	5.67	±0.214554
	T6	4.54	4.2	4.49	4.41	±0.183576
Brazilian	T0	1.56	1.57	1.49	1.54	±0.043589
	T1	2.57	2.89	2.95	2.8	±0.204287
	T2	3.34	3.2	3.5	3.35	±0.150111
	T3	4.59	4.78	4.26	4.54	±0.263122
	T4	5.57	5.4	5.89	5.62	±0.248797
	T5	6.53	6.89	6.39	6.6	±0.257941
	T6	5.75	5.57	5.79	5.7	±0.117189

	T0	T1	T2	T3	T4	T5	T6
BAP mg/L	0	1	2.5	5	1	2.5	5
IAA mg/L	0	0.5	0.5	0.5	1	1	1

Appendix 2: Mean values of different banana genotypes for longest shoot length

Varieties	Treatments	Replication 1	Replication 2	Replication 3	Mean	Standard deviation
Wiallium-8818 hybrid	T0	3.325	3.233	3.212	3.26	±0.060103
	T1	5.137	5.467	5.323	5.31	±0.165445
	T2	6.913	6.768	6.767	6.82	±0.084006
	T3	10.235	10.515	10.434	10.39	±0.144084
	T4	12.127	12.272	12.196	12.2	±0.072528
	T5	11.189	11.818	11.325	11.44	±0.330955
	T6	10.234	10.324	10.143	10.23	±0.0905
Pisang	T0	3.128	3.182	3.821	3.38	±0.385462
	T1	3.651	3.612	3.519	3.59	±0.067816
	T2	4.1452	4.322	4.153	4.21	±0.0999
	T3	5.741	5.645	5.675	5.69	±0.049112
	T4	6.313	6.341	6.458	6.37	±0.076918
	T5	4.957	4.676	4.206	4.61	±0.379443
	T6	3.321	3.769	3.873	3.65	±0.293321
Brazilian	T0	2.563	2.622	2.529	2.57	±0.047057
	T1	3.757	3.919	3.837	3.84	±0.081002
	T2	4.693	4.654	4.817	4.72	±0.085114
	T3	6.965	6.768	7.231	6.99	±0.232355
	T4	8.198	8.256	8.359	8.27	±0.081541
	T5	5.479	5.326	5.273	5.36	±0.106969
	T6	4.532	4.645	4.459	4.55	±0.093714

	T0	T1	T2	T3	T4	T5	T6
BAP mg/L	0	1	2.5	5	1	2.5	5
IAA mg/L	0	0.5	0.5	0.5	1	1	1

Appendix 3: Mean values of different banana genotypes for fresh weight

Varieties	Treatments	Replication 1	Replication 2	Replication 3	Mean	Standard deviation
Wiallium-8818 hybrid	T0	2.517	2.407	2.543	2.49	±0.072194
	T1	3.329	3.234	3.235	3.27	±0.054562
	T2	4.159	4.157	4.158	4.16	±0.001
	T3	5.632	5.432	5.765	5.61	±0.16762
	T4	8.987	8.784	8.542	8.77	±0.222785
	T5	12.989	13.016	13.237	13.08	±0.13606
	T6	14.571	14.086	14.524	14.39	±0.267481
Pisang	T0	2.721	2.876	3	2.82	±0.139787
	T1	3.552	3.725	3.873	3.72	±0.160662
	T2	5.764	5.823	5.745	5.78	±0.040673
	T3	9.765	9.872	10.542	10.06	±0.421125
	T4	10.982	10.989	11.21	11.06	±0.129662
	T5	13.563	13.893	13.764	13.74	±0.166304
	T6	15.267	15.652	15.432	15.45	±0.193154
Brazilian	T0	3.451	3.343	3.784	3.53	±0.229867
	T1	3.765	3.733	3.785	3.76	±0.02623
	T2	4.132	4.132	4.342	4.20	±0.121244
	T3	5.634	5.345	5.458	5.48	±0.14564
	T4	6.276	6.367	6.562	6.40	±0.146118
	T5	8.175	8.175	8.153	8.17	±0.012702
	T6	9.104	9.004	9.035	9.05	±0.051189

	T0	T1	T2	T3	T4	T5	T6
BAP mg/L	0	1	2.5	5	1	2.5	5
IAA mg/L	0	0.5	0.5	0.5	1	1	1

Appendix 4: Mean values of different banana genotypes for leaves per shoot

Varieties	Treatments	Replication 1	Replication 2	Replication 3	Mean	Standard deviation
Wiallium-8818 hybrid	T0	2.5	2.7	2.5	2.57	±0.11547
	T1	3.4	3.9	3.1	3.47	±0.404145
	T2	4.5	4.9	4.2	4.53	±0.351188
	T3	5.6	5.2	5.8	5.53	±0.305505
	T4	6.2	6.6	5.9	6.23	±0.351188
Pisang Brazilian	T0	2.5	2.6	2.7	2.6	±0.1
	T1	3.1	3.3	3.3	3.23	±0.11547
	T2	4.1	3.9	4.2	4.07	±0.152753
	T3	4.9	5.1	4.7	4.9	±0.2
	T4	5.6	5.8	5.4	5.6	±0.2
	T0	2.5	2.2	2.8	2.5	±0.3
	T1	2.9	3.1	2.8	2.93	±0.152753
	T2	3.9	3.9	3.8	3.87	±0.057735
	T3	4.8	4.5	4.4	4.57	±0.208167
	T4	5.4	5.3	5.1	5.27	±0.152753

	T0	T1	T2	T3	T4
IAA mg/L	0	0.5	0.5	1	0
NAA mg/L	0	0	1	1	0.5

Appendix 5: Mean values of different banana genotypes for roots per shoot

Varieties	Treatments	Replication 1	Replication 2	Replication 3	Mean	Standard deviation
Wialium-8818 hybrid	T0	2.2	1.9	2.4	2.17	±0.251661
	T1	2.4	2.1	2.6	2.37	±0.251661
	T2	2.9	3.2	3.2	3.10	±0.173205
	T3	4.1	3.9	3.8	3.93	±0.152753
	T4	3.9	3.5	3.7	3.70	±0.2
Pisang	T0	2.9	2.7	2.1	2.57	±0.416333
	T1	3.2	3.2	3.3	3.23	±0.057735
	T2	4.7	4.5	4.6	4.60	±0.1
	T3	5.6	5.8	5.1	5.50	±0.360555
	T4	4.7	4.5	4.8	4.67	±0.152753
Brazilian	T0	2.3	2.5	2.4	2.40	±0.1
	T1	2.7	3.2	2.9	2.93	±0.251661
	T2	3.5	3.8	4.2	3.83	±0.351188
	T3	4.5	4.8	4.4	4.57	±0.208167
	T4	4.3	4.4	4.2	4.30	±0.1

	T0	T1	T2	T3	T4
IAA mg/L	0	0.5	0.5	1	0
NAA mg/L	0	0	1	1	0.5

Appendix 6: Mean values of different banana genotypes for longest root length

Varieties	Treatments	Replication 1	Replication 2	Replication 3	Mean	Standard deviation
Wlallium-8818 hybrid	T0	4.153	4.157	4.152	4.15	±0.002646
	T1	4.212	4.528	4.725	4.49	±0.25879
	T2	4.562	4.758	4.896	4.74	±0.167837
	T3	4.397	4.346	4.397	4.38	±0.029445
	T4	4.562	4.234	3.892	4.23	±0.335024
Pisang	T0	4.293	4.231	4.301	4.28	±0.038314
	T1	5.352	5.826	4.924	5.37	±0.451195
	T2	6.315	6.312	6.132	6.25	±0.1048
	T3	6.193	6.192	6.322	6.24	±0.07479
	T4	5.295	5.259	5.283	5.28	±0.01833
Brazilian	T0	3.987	4.231	3.462	3.89	±0.392964
	T1	5.195	5.163	5.184	5.18	±0.016258
	T2	5.528	5.985	5.752	5.76	±0.228515
	T3	5.762	5.762	5.762	5.76	±0.102763
	T4	4.873	4.873	4.873	4.87	±0.305505

	T0	T1	T2	T3	T4
IAA mg/L	0	0.5	0.5	1	0
NAA mg/L	0	0	1	1	0.5