In vitro protocol optimization for mass propagation of cooking banana (*Musa Sapientum L.*) (Matoke and Nijiru cultivars)

By: Adugna Mosissa Kajela

A Thesis Submitted to Applied Biology Department, Adama Science and Technology University, in Partial Fulfillment of the Requirement for the Degree of Master of Science in Applied Biology (Specialization in Biotechnology)

School of Applied Natural Sciences

Office of Graduate Studies

Adama Science and Technology University

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Co-Advisor: Mr. Abel Debebe

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Approval of Board of Examiners

We, the undersigned, members of the Board of Examiners of the final open defense by Adugna Mosissa have read and evaluated his thesis entitled “In vitro protocol optimization for mass propagation of cooking banana (*Musa Sapientum*L.) (Matoke and Nijiru cultivars)” and examined the candidate. This is, therefore, to certify that the thesis has been accepted in partial fulfillment for the requirement of the Degree, Master of Science in Applied Biology (Biotechnology).

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Declaration

I hereby declare that this MSc thesis is my original work and has not been presented for a degree in any other university, and all sources of material used for this thesis have been properly acknowledged.

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This MSc thesis has been submitted for examination with my approval as thesis advisor.

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Advisor's approval sheet

To: Applied biology department

Subject: Thesis Submission

This is to certify that the thesis entitled In vitro protocol optimization for mass propagation of cooking banana (*Musa Sapientum*L.)Matoke and Nijiru cultivars) submitted to the Department of Applied Biology in partial fulfillment of the requirements for the degree of Master's of Science in Applied Biology (Biotechnology), by Adugna Mosissa, ID No: A/PR15395/10, under my supervision. Therefore, I recommend that the student has fulfilled the requirements and hence hereby he can submit the thesis to the Department.

_____ _____ _____

Name of major Advisor Signature Date

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Table of Contents

List of Figures

List of Tables

List of Abbreviation and Acronym

ANOVA= Analysis Of Variance

BAP=6-Benzyl-9-(2-tetrahydropyranylamino purine)

BBTV= banana bunchy top virus

BXW= banana xanthomonas wilt

CK= cytokin

CRD= Complete Randomized Design

FAO=Food and Agriculture Organization

FAOSTAT= Food and Agriculture Organization Statistics

FYM=Farm Yard Manure

IBA= Indolebutric acid

L=Liter

MARC= Melkessa Agricultural Research Center

mm= millimeter

MS= Murashig and Skoog's

NAA= Neapthline acetic acid

NR=Number of root

NSH= Number of shoot

PPF= photosynthetic photon flux

PPM=Part per million

RL=Root Length

SAS= statistical analyses

SPP= species

Abstract

Edible bananas (Musa spp.) are the major staple food for rural and urban consumers in the tropical and subtropical countries and an important source of rural income. The study was aimed to develop a simple, comprehensive and efficiently repetitive protocol for micropropagation of banana (Musa sapientum L.). For this experiment the banana cultivars used were Matoke and Nijiru. Those two cultivars were taken from the field and grew under green house for three month and taken to the laboratory for initiation. After two month of the culture initiation the developed culture shoot proceed to the shoot multiplication and rooting media. Using shoot meristem and increase the production and productivity of the farmers. The shoot tip initiation response survival of75% and 68% were obtained from explant cultured on MS medium supplemented with 3.0 mg/l BAP alone for both banana cultivars Matoke and Nijiru respectively. The highest number of shoots and shoot multiplication rate was obtained on MS medium supplement with a combination of BAP and IAA at concentrations of 3mlBAP+0.6ml IAA mg/l (14.2) for Matoke cultivar. For Nijiru cultivar most productive and produced maximum number of shoots at concentration of BAP and IAA; 3mlBAP+0.2mlIAA (11.6) followed by 3mlBAP+0.4mlIAA. Antibiotic (cefotaxime) was used to check the endogenously born bacterial contamination. The proliferated shoots were excised and transferred to different root induction media, which resultantly showed that MS media supplemented with IAA NAA and IBA, were the most efficient root inducing media. Rooted plantlets after primary and secondary hardening were transferred to the green house in which were successfully acclimatized.

Keywords: MS medium, Shoot multiplication, Secondary hardening, Proliferated shoots

1. INTRODUCTION

Bananas *(Musa Sapientum L.)* are the most important food crop in the world, with a production approximating 102 million 6per year. It is the world's most widely known and distributed fruit, eaten either raw or cooked, and may be processed into starch, chips, beer, vinegar or dried products. Banana are rich in energy (128kcal/100g), and vitamin C and A. Banana fruits have a very high content of potassium (k) and a wide K/Na ratio, imparting a protective effect of K against excessive Na intake in diets (FAO, 2012).

Edible bananas (Musa spp.) are the major staple food for rural and urban consumers in the tropical and subtropical countries and an important source of rural income. The genus Musa (family *Musaceae*) originates in Asia (Simmonds, 1962). Cultivated banana is derived from two diploid species of genus *Musa*. *M. acuminate* (Malaysia) and *M. balbiciana* (India) parent genomes (Stover & Simmonds, 1987; Simmonds, 1962; George *et al.*, 2000). Banana is a good source of carbohydrates, proteins, vitamins and minerals. Many pest and diseases (especially viral diseases i.e. banana mosaic virus) constrain banana production which resulted in serious consequences for environment through the application of pesticides. Thus, major constraints in the banana production system are the non-availability of disease-free, true-to-type planting material, low fertility due to triploidy, slow propagation and long time span from one generation to the next generation. Classical breeding is difficult Because of its high degree of sterility and polyploidy of the edible varieties (Stover & Simmonds, 1987).

Bananas belong to group of crops which are normally propagated through vegetative parts of the plant because almost all cultivated banana cultivars are triploid, seedless, or seed sterile. The materials used for conventional propagation include corms, large and small suckers, and sword suckers (Cronauer & Krikorian, 1984; Arias, 1992; Haq and Dahot, 2007). Mass propagation of selected genotypes, somaclonal variation techniques, genetic engineering and other biotechnological applications can be utilized for banana crop improvement which is based on reliable plant regeneration protocols. Tissue culture also plays a vital role in the distribution of germplasm, conservation, safe exchange of internal planting material and rapid propagation of newly selected hybrid cultivars. Several researchers have reported the regeneration of Musa spp. via micro propagation (Cronauer & Krikorian, 1986; Jarret, 1986; Diniz *et al.*, 1999; Nauyen &

Kozai, 2001; Krishnamoorthy et al., 2001; Kagera et al.,., 2004; Muhammad et al, 2004; Roels et al., 2005; Madhulatha et al., 2004). But, propagation percentage and repeatability of the method are matters of concern which ultimately need a comprehensive, repeatable and applied method for a wide range of genotypes to facilitate disease free production of banana crop on commercial scale.

For in vitro micropropagation of banana, bacterial contamination is a big problem. Although initially surface sterilization works, latter on microbial contamination at the base of the explant appears within 7 to 15 days after inoculation. Huge number of explants is destroyed in the culture due to endogenous bacteria (Habiba et al., 2002). The present study suggests a rapid banana multiplication protocol from shoot meristem by using a medium with optimized concentration of auxins/cytokinins. Many reports are available on in vitro propagation with complicated protocols but less shoot proliferation percentage which eventually yield less number of regenerated plants per culture.

Ethiopian farmers had long time experience of planting and producing this fruit. But they didn't obtain plant materials that free from contaminants. So, to provide farmers with desired planting materials is important through optimizing in vitro protocol for selected cultivars. Bananas are asexually propagated by either separating the daughter suckers from the mother plant or tissue culture. Tissue culture, unlike the sucker-derived planting material has various advantages. These include producing uniform planting materials clean, disease-free materials and many plants in a small space. It offers mass propagation and clean planting material. They are cheaper to transport than conventional suckers and the coupling with virus indexing allows for safe movement, and exchange and conservation of germplasm.

In addition, bananas produced using tissue culture technology are reported to be more vigorous, higher yielding and produce better quality fruits than those produced by conventional means (De Langhe (1985). Inspite of these advantages, tissue culture technology remains unknown or under exploited in Africa (Murungikahangi.E, 2010). So, to exploit the merits of this technology, it is inevitable to do protocol optimization work. For this plant, growth regulators are crucial for in vitro regeneration of crop plants in artificial medium. Cytokines helps in shoot proliferation and auxins helps in rooting of proliferated shoots. However, the requirement of cytokines and auxins depends on the variety of banana and culture condition (Alamin, 2009). Plantains-

cooking banana which might play vital role in food security of Ethiopia. Thus developments of a protocol for shoot tip culture of cooking banana (Matoke and Nijiru cultivars) are primarily important to increase production and productivity for banana product to insure food security of the country.

1.1 Statement of the problem

Bananas play an important role in the food security of developing countries, especially in sub-Saharan Africa (FAOSTAT, 2010). They are cultivated in over 120 countries covering about 10 million ha, with an annual production of 130 million tons (FAO, 2009). In Africa, banana and cooking banana provide more than 25% of the food energy requirements for more than 100 million people, of 20 million are from East Africa alone (FAOSTAT, 2004).

Banana is the most produced fruit in Ethiopia for food and commercial purposes. However, there are constraints in the production of banana. Among the banana production constraints, low propagation, low quality and quantity of yield. There is a need for biotechnological applications to improve the production of disease free, develop micropropagation protocols and quality plantlets. Therefore, development of a reliable and efficient regeneration and transformation protocol of farmer-preferred cultivars using micro propagation and meristematic tissues as explants is a prerequisite for mass propagation of banana.

1.2. Significance of the study

No doubt, bananas remain one of the most studied plants in the field of plant biotechnology. The huge economic importance and the inherent problems associated with conventional propagation of bananas makes the species one of the highly prioritized research crops globally **(**Nwauzomaetal, 2002**)**. In addition, an increase in demand by the world growing population implies the need for more planting materials of high quality (free of pest and diseases).

Generally, the importance of this study is to develop an optimized micropropagation protocol which could be used in different labs for the rapid micropropagation and dissemination of elite banana cultivars. The work will have a significant impact in addressing food security issues by increasing production and productivity of the banana

1.3. Objectives

1.3.1 General objective

- To develop rapid micropropagation protocols of cooking banana (*Musa Sapientum*L.) (Matoke and Nijiru cultivars).

1.3.2 Specific objectives

- To developoptimized protocol for initiation cooking banana(Matoke and Nijiru) cultivars,
- To develop optimized protocol for shoot multiplication of cooking banana (Matoke and Nijiru) cultivars
- To develop optimized protocol for rooting and rapid mass propagation using shoot culture for quantity and quality improvement of bananas and growth under green house

2. LITERATURE REVIEW

2.1. Tissue culture of banana

Sengar*et al.* (2010) concluded that micropropagation of bananas remain as the next best alternative of natural regeneration with tremendous potential for production of quality planting material. Thus, the farmers are benefited by tissue-cultured plants because of ease of multiplication of their variety of choice and also suggested that micropropagation is user-friendly technique which does not require much expertise and is suitable for adoption by small and marginal farmers and success of micro propagation depends on method, variety and price of initiation media. Further, Gitonga*et al*., (2010) evaluated a micropropagation protocol for local banana (*Musa* spp., Muunju landrace) in Kenya as an alternative to reduce the unit cost of tissue culture micro propagation. Ngomuo*et al.*, (2014) reported that the planting materials of banana obtained through conventional methods (suckers) do not meet the increasing demand for planting and they are of poor quality.

Gray and Daniels (2015) described micropropagation as the practice of growing plants like banana from meristematic tissue or somatic cells of superior plants *in vitro*. Cultures are established on a separate initiation medium, which has a lower concentration of cytokinin than the multiplication medium, to which cultures are subsequently transferred (Jarret*et al.*, 1985; Novak *et al.*, 1989). Akbar and Roy (2006) cultured banana explants on MS medium supplemented with 0.5 mg/l of BA, Kn and NAA and found that addition of 10% coconut water to the medium resulted in increased number of differentiated shoots per culture. It was also found that acclimatization and transplantation performances of plants was superior for those rooted in liquid medium compared to those rooted in solid medium.

Madhulata*et al.*, (2006) studied the effect of carbon sources (sucrose, glucose, fructose and mannitol) on *in vitro* propagation of banana and found the highest frequency of shoot proliferation on the medium containing sucrose compared to all other carbon sources. It was also reported that MS medium supplemented with sucrose and glucose combination (1:1) at the concentration of 30 gm/l showed the optimum shoot proliferation. Further, Feng*et al.*,(2007) optimized medium for *in vitro* proliferation of banana and found that 40 gm/l sugar not only facilitated proliferation of buds but also controlled proportion of buds with leaf sheaths and

increased the available bud index. The effect of nutrient medium constituents on growth and development of banana plantlets produced *in vitro* was evaluated by Hussein (2012). He found that the medium with sucrose at 30% supplemented with 0.4mg/l BAP was the most optimum for banana shoot tip culture as expressed by better growth vigour, plantlet height, fresh weight as well as stronger shoot and root system. Ahmed *et al.* (2014 a) investigated the effect of different carbohydrate source, pH and supporting media on *in vitro* rooting of banana plantlets using MS medium with 0.1 mg/l IBA and activated charcoal. Sucrose in the medium remarkably influences the rooting of plantlets. In the absence of sucrose, culture could not survive after 3 weeks of incubation. In the sucrose containing media, 30 g/l gave the best result. Out of different pH levels tested, minimum time for root initiation with longest length of root was obtained on pH 5.5.

2.2. Explant choice and sterilization

Sharrock (1992) reported that in vitro cultures of both pineapple and plantain can be initiated from any growing point of the plant. Thus, dormant buds and shoots that are unsuitable for conventional propagation Rates of multiplication *in vitro* are much higher than those achievable in conventional propagation systems. Jyothi*et al.,* (1993) cultured shoot tips, eye bud or floral apex explants of banana cv. Red banana on semi-solid MS medium supplemented with various growth regulators.

Muhammad *et al*.,(2004) reported that banana shoot tips derived from different suckers can be used to study multiplication rate in banana. Further, Perez and Hooks (2008) reported that micro propagating banana through shoot tip is the main method used for fast propagation of banana plants. Matsumoto *et al.*,(2010) reported that the protoplast culture and somatic hybridization are a feasible technique of micropropagation and to support the genetic improvement of banana. Ahirwar *et al*.,(2012) used male inflorescence tip explants and shoot tip of banana for studying micropropagation in banana. Goswami and Handique (2013) studied the effect of three different sizes of explants (5, 10 and 20 mm) on the establishment of banana in micropropagation. Three cultivars (Amritsagar, Malbhog and Chenichampa) were used for the study. They found that larger explants (20 mm) responded well with regard to survival of explants, days to swelling and greening of explants, emergence of leaf and days to multiple bud initiation under *in vitro*

condition as compared to smaller explants. Culture condition Explants should be free from surface contaminants to accomplish growth and development under aseptic conditions and this is achieved by surface sterilization. The sterilization treatment should be so selected that it kills the microbes without affecting the plant tissues adversely. Contamination in tissue culture may be caused by endogenous bacteria that escape initial disinfection or by microorganisms introduced during tissue culture manipulations. There are several antibiotics which are successful in controlling bacterial contaminants in banana tissue culture.

Oliveira *et al.*,(2000) evaluated two methods of banana explant disinfection and the use of 3 indicator culture media for banana micropropagation. Shoot tips of the cultivar Pioneira were disinfected by (1) immersion in 80% alcohol for 2 min and in (2) sodium hypochlorite (2% active chlorine) with 4 drops of Tween-20 for 10 min, with shaking. The contamination observed was only of bacterial nature. Lima and Moraes (2006) evaluated the bacterial contamination control methods in banana explants cv. Caipira using NaOCl, rifampicin antibiotic and their combinations. The best treatment for explants was the immersion in 1% (v/v) NaOCl for 10 minutes, followed by immersion in 300 mg/l rifampicin for 20 minutes. After contamination, the best treatment was the immersion in 1% NaOCl for 10 minutes, followed by immersion in 300 mg/l rifampicin for 24 hours in the dark.

The disinfectants widely used are sodium hypochlorite (which dates back to the mid-18th century), calcium hypochlorite, ethanol (or isopropyl alcohol), mercuric chloride, hydrogen peroxide, silver nitrate and bromine water to enhance effectiveness in sterilization procedure, a surfactant like Tween-20 is frequently added to the sterilizing solution (and in some laboratories a mild vacuum is applied during the procedure); in general, the sterilizing solutions containing the explants are continuously stirred during the sterilization period (Oyebanji, *et al*., 2009). Jing-Yan *et al.*,(2011) studied the effects of different disinfectant on explants. Mercuric chloride and 0.2% sodium hypochlorite solutions were used to sterilize the sucker buds of Longxuan banana which were used as explants. Sodium hypochlorite showed better effects than mercuric-chloride on the disinfection of explants, the sterilization rate was 90.47% and the explants grew well without any intoxication.

Goswami and Handique (2013) did the sterilization of explants by firstly treating with savlon for 15 minutes followed by sterilizing with a mixture of 2% NaOCl+1 gm/l Captan or DithaneM-45 and rifampicin (0.1%) for 45 minutes with Tween-20. Thereafter, quick dipping of explants (15 sec) in 70% alcohol. On laminar air flow cabinet explants treated with NaOCl (0.5-1%) for 15 minutes followed by treatment with 0.1% HgCl2 for 7 minutes proved better than the other treatments. Bohra*et al.,* (2014) studied the aseptic culture establishment using antibiotics with reference to their efficiency and phytotoxicityin difficult-to-establish native Ney Poovan banana (*Musa* AB). They standardize antibiotic supplement for obtaining aseptic cultures in native banana variety Elakki Bale. Of the different antibiotics and combinations tried, chloramphenicol was found to give 100% aseptic cultures followed by rifampicin + chloramphenicol, rifampicin and chloramphenicol + streptocycline combination. Rifampicin was found to cause least phytotoxicity. The plantlets grew normally in subsequent cycles and 96.3 % plantlets could survive up on transfer to ex vitro conditions.

Anbazhagan*et al*., (2014) did surface sterilization of the explants primarily by rinsing in tap water for 30 minutes followed by gently rinsing with 70% ethanol for 60 seconds and with 5% sodium hypochlorite solution for 10 minutes. Further, sterilization procedures were carried out in laminar air flow chamber by using 0.1% HgCl2 for 5 minutes. Condition of culture room Light, temperature and humidity conditions provided inside the culture room plays a significant role in success of an *in vitro* technique. The light intensity, quality and duration are the major factors affecting the growth of *in vitro* culture (Murashige, 1974, 1977). Murashige (1977) found a 16 h photoperiod satisfactory for a wide range of plant species. Relative humidity of the culture room is adjusted to 55±5%. Banana shoot tip cultures are incubated at an optimal growth temperature of 28± 2ºC in a light cycle of 12-16h with a photosynthetic photon flux (PPF) of about 60µE/m2/s.

2.3 Effect of different type and concentration of growth hormone on shoot initiation

Invitro culture of banana shoot tips resulted hard meristematic ball like structure in initiation media containing different concentrations of BAP and IAA. The cultured shoot tip turned brown in color from the initial creamy white in a few days after inoculation (Muhammad Munir Iqbal, 2013). Four weeks later, the external leaf primordial of explants turned green and globular hard

coat mass grew from which adventitious plantlets were developed as reported by Muhammad *et al*., (2004) using BAP. Cronauer and Krikorian (1984) and Vuylsteke (1998) also reported BAP as the most commonly preferred cytokinin used in banana tissue culture. Furthermore, Al-Amin *et al*., (2009) observed the color change of culture meristems to brown in 4 to 5 days and a development of a green hard ball like structure after 30 to 35 days of inoculation.

2.4 Effect of different type and concentration of growth hormone on shoot multiplication

According to Cronauer and Krikorian, (1984) reported the multiple shoots could be produced from sliced shoot tips of banana and plantain. Auxins and cytokinins have been reported as the most frequently used PGRs for banana micropropagation (Vuylsteke, 1998). Adenine-based cytokinin particularly BAP is the most commonly preferred cytokinin to affect shoot multiplication rate in several *Musa* spp. (Cronauer and Krikorian, 1984; Vuylsteke, 1998). Differences in rate of multiplication of different *Musa* genotypes under *in vitro* conditions have also been reported (Vuylsteke, 1998; Muhammadi*et al*., 2004). It has been reported that multiple shoots could be produced from sliced shoot tips of banana and plantain (Cronauer and Krikorian, 1984).Auxins and cytokinins have been reported as the most frequently used PGRs for banana micropropagation (Vuylsteke, 1998). Adenine-based cytokinin particularly BAP is the most commonly preferred cytokinin to affect shoot multiplication rate in several *Musa* spp. (Cronauer and Krikorian, 1984; Vuylsteke, 1998).

Differences in rate of multiplication of different *Musa* genotypes under *in vitro* conditions have also been reported (Vuylsteke, 1998; Muhammadi*et al*., 2004). Srangsam and Kanchanapoom (2003) observed yellow friable calluses of banana (*Musa* spp.) 'Gros ichel', cultured on Murashige and Skoog (MS) solid medium supplemented with 2, 4 dichlorophenoxy acetic acid (2, 4-D) and coconut water (CW). Small spherical, compact calluses were formed. Friable calluses were transferred to half-MS liquid media supplemented with 1.5 mg/l 2, 4-D or 1.5 mg/l 2, 4-D in combination with 5% CW or without 2, 4- D and CW. No shoots were produced from these media. Embryogenic calluses were induced followed by subculture the spherical, compact calluses to half-MS solid medium in the presence of thidiazuron (TDZ). These embryogenic calluses gave rise to shoots on MS germination medium containing 2.0 mg/l α-naphthalene acetic acid (NAA) and 1.0 mg/l 6-benzyladenine (BA).

Muhammad *et al.,* (2004) cultured the shoot tips of banana cv. Basrai on Murashige &Skoog basal medium supplemented with 5.0 mg/l BAP and he recorded on the average, 124 plants produced from each shoot tip after five sub culturing. Al-Amin *et al*., (2009) studied the effect of different concentrations of BAP and NAA on plant regeneration and shoot multiplication of banana cv. BARI banana-I. Highest shoot proliferation, longest shoot production, maximum no. of leaves and longest leaves were found at concentration of 7.5 mg/l BAP + 0.5 mg/l NAA. Jing-Yan *et al*. ,(2011) added different hormonal combinations (0-6.0 mg/l 6-BA and 0.1-0.2 mg/l NAA) in MS medium to induce the adventitious buds in banana cv. Longxuan and to allow its multiplication. The best induction and multiplication medium for adventitious buds was found at MS + 3.0 mg/l 6-BA and MS + 4.0 mg/l 6-BA + 0.2 mg/l NAA, respectively.

Aremu *et al.*,(2012) studied the effects of five topolins (metaTopolin=*m*T; metaTopolinriboside= *m*TR; metaMethoxytopolin= MemT; metaMethoxytopolinriboside= MemTR and metaMethoxyt opolin 9tetrahydropyranyl = MemTTHP) on shoot regeneration of micropropagated 'Williams' bananas and compared to benzyladenine (BA). 30µM *m*T resulted in the highest number of shoot s (7.3±1.0). Unlike other CK treatments requiring higher concentrations, optimum mean shoot number per explant was attained at the lowest concentration in Me*m*T and Me*m*TTHP (10µM) treatments. In terms of abnormality index, *m*TR regenerated plantlets were of the best quality across all the CKs tested. Mondal*et al.,* (2012) studied the effect of coconut water and ascorbic acid on shoot regeneration in banana variety Dwarf Cavendish. They inoculated the shoot tips on MS medium containing BAP (Benzyl Amino Purine) 5.0 mg/l supplemented with coconut water in various concentration (0, 50, 100, 150 and 200 ml/l) and with various concentrations (0, 25, 50, 75 and 100 mg/l) of ascorbic acid, respectively.

As the concentration of coconut water and ascorbic acid was increased up to 100 mg/l and 50 mg/l respectively greater frequency of explant in shoot regeneration, no. of shoots regenerated per explant and shoot length was observed. Sipen and Davey (2012) studied the different concentrations of N6 benzylaminopurine (BAP) and Indole Acetic Acid (IAA) for their effect on shoot multiplication and plant regeneration of the Malaysian banana cultivars Pisang Mas, PisangNangka, PisangBerangan and PisangAwak. Maximum shoot was produced on medium supplemented with BAP at 5 mg/l (PisangNangka), 6 mg/l (Pisang Mas and PisangBerangan)

and 7 mg/l (PisangAwak) with 0.2 mg/l IAA. Further, Ahirwar*et al*. (2012) took different concentration of BAP (0-10 mg/l), Kinetin (0-10mg/l), NAA (0.3-0.5mg/l) and different combination of BAP (0-10 mg/l) and NAA (0.3- 0.5 mg/l). Highest frequency of shoot regeneration (52.25), number of shoots regenerated per explants (3.25) and shoot length (4.69) was found at BAP concentration of 5 mg/l, Kinetin concentration of 5 mg/l and combination of 7.5 mg/l BAP + 0.3 mg/l NAA.

The addition of 5 mg/l BAP was found better than Kinetin for shoot development from shoot tip or male inflorescence tip explants. Rahman*et al*.,(2013) investigated the best plant growth regulators for shoot proliferation and multiplication for cultivar Agnishwar. Among different types and concentration of cytokinins viz. 6-benzylaminopurine (BAP), kinetin (KIN), N6 - (2-isopentyl) adenine (2iP) tested for multiplication of shoot; maximum multiplication (95%) was obtained in MS medium containing 4.0 mg/1 BAP. The highest average number of shoots for each explant (5.9) was found in MS medium fortified with 4.0 mg/l BAP while maximum elongation of shoot (4.9 cm) was observed in MS medium containing 5.0 mg/l BAP. Ramachandran and Amutha (2013) carried out research work on Cavendish Dwarf variety of banana. Murashig and Skoog's basal medium supplemented with 4 mg/l BAP and 0.2 mg/l NAA was found to be most suitable combination for shooting.

Further, for multiplication combination of BAP 5mg/l+ NAA 0.3mg/l was found best while Ahmed *et al.* (2014) found MS medium supplemented with BAP 4.00 mg/l + IAA 2 mg/l best for explant establishment and shoot multiplication of banana cv. Grand Naine. Shiv Shankar *et al.*, *(*2014) did mass propagation of banana (*Musa* spp.) cv. Grand Naine through direct organogenesis by using PGRs Benzyl Adenine Purine and Kinetin. Benzyl Adenine Purine (BAP) in five different concentrations (control, 2.0, 4.0, 6.0, 8.0 and 10.0 mg/l) were used for shoot proliferation and differentiation and shoot multiplication rate. The study revealed that medium supplemented with BAP 4.0 mg/l produced greater number of shoots (55) and longer sh oot (3.0±0.012cm) when compared with other treatments. Reddy *et al*., (2014) studied the effect of diverse concentration of 6-benzylamino purine (6-BAP) on shoots induction of *Grandnaine* plantlets (*Musa* spp). Anbazhagan*et al.*, (2014) cultured shoot tips of *Musa* spp. on Murashige and Skoog (MS) medium supplemented with different concentrations of BAP, KIN

and IAA both in individual and in combined form and the best results were obtained from MS medium supplemented with BAP + IAA at the concentration of 3.0 mg/l and 0.5mg/l respectively. Jamir and Maiti (2014) studied the effect of various levels of cytokinin and auxin for *in vitro* regeneration of banana cultivars Grand Naine and Jahaji. They tested various concentration of BAP (0-6.5 mg/l). 4.5 mg/l BAP was found as the best concentration in induction of highest no. of buds (an average of 7.05 and 7.2) with highest mean length of 0.65 cm and 0.7 cm of shoots. But, the shoot elongation was maximum at lower concentration of BAP (1.5mg/l).

Research works were conducted by Shashikumar*et al*., (2015) with BAP, TDZ and coconut water at various concentrations and recorded high frequency of shoot initiation (93.33) at 5 mg/l BAP. The synergetic effect of BAP (4 to 6 mg/l), TDZ (0.1 to 1.2 mg/l) and coconut water (0.1 to 0.9 ml/l) induced multiple shoot buds and this was optimum at the concentration of 5 mg/l BAP, 0.5 mg/l TDZ and 0.5 ml/l coconut water with 15.90 ± 1.66 frequency of shoots per propagated. Suman and Kumar (2015) did the micropropagation of banana cv. Malbhog on Murashige and Skoog (MS) medium supplemented with different concentrations and combinations of Indole Acetic Acid (IAA) and Benzyl Amino Purine (BAP). This combination resulted in differentiation of adventitious shoots. The maximum differentiation of shoots (92.05 %) was observed on MS medium with 0.57 µm IAA + 17.74 µM BAP. The number of shoots per culture was 16.75. The subculture of differentiated shoots on the same medium resulted in further differentiation (91.97 %) of more than 15 shoots per culture.

2.5 Study of different concentration of growth hormones on rooting

Al-Amin *et al.* (2009) used half strength MS medium supplemented with different levels of IBA (0, 0.5, 1.0 and 1.50 mg/l) and IAA (0, 0.5 and 1.0 mg/l) for root initiation in banana cv.BARI-1. The highest number of roots (6.50) and longest length of root (5.88 cm) was obtained on 0.5 mg/l IAA + 0.5 mg/l IBA. Sipen and Davey (2012) found that half strength MS medium fortified with 1 mg/l NAA was suitable for root regeneration from scalps in Malaysian banana cultivars PisangMas, PisangNangka, PisangBerangan and PisangAwak. They found maximum of 7 roots from cv. PisangNangka and PisangBerangan and mean root length was also maximal (4.5 cm) in the latter two cultivars. Rai*et al*. (2012) inoculated multiplicities shoots of banana cv. Grand

Naine (G-9) on rooting media incorporated with either IBA or NAA (0.25, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/l) and Charcoal (2 g/l) for root induction. IBA (2 mg/l) and Charcoal (2 g/l) produced maximum number of roots (8.5) with a lot of root hairs. Rahman*et al*., (2013) investigated the best plant growth regulator for induction of roots in banana cv. Agnishwar. Among different types and concentration of auxin viz. Indole-3-Butyric Acid (IBA) and α-naphthalene acetic acid (NAA), IBA at 1.0 mg/l was found most suitable for rooting of shoot (96% rooting). Ramachandran and Amutha (2013) transferred cultured plants of banana cv. Cavendish Dwarf into media containing activated charcoal and hormone (NAA-1.5 mg/l). Ahmed *et al*., (2014) obtained rooting on MS (half strength) medium fortified with IBA 1.00 mg/l and activated charcoal 200mg/l.

Aremu*et al*., (2014) conducted rooting experiments involving the use of indole-3-acetic acid, indole-3-butyric acid, naphthalene acetic acid, smoke–water (SW) and karrikinolide (KAR1). Significantly increased number and length of roots were obtained in SW and KAR1 compared to the control. Overall, when compared to BA, the use of topolin demonstrated higher mean shoot number per explant (Me*m*T and Me*m*TTHP) at lowest CK concentrations and the ease of rooting during the shoot proliferation phase (Me*m*TTHP). Shiv Shankar *et al*. (2014) assessed the development of roots in regenerated shoots of *Musa* spp. cv. Grand Naine by treating the shoot with five levels of kinetin (control, 0.5, 1.0, 1.5, 2.0 and 2.5 mg/l) supplemented in MS medium. Root development was not observed in the medium devoid of hormone. Whereas, increased number of roots (4.0), length of roots (6.0 cm) and length of shoots (6.5 cm) was observed in medium with 1.0 mg/l kinetin. Anbazhagan*et al*. (2014) found that best root formation (96%) and root number / explant (11.80) of *in vitro* developed shoots of *Musa* spp. could be achieved on half strength MS medium supplemented with IBA at 1.0 mg/l.

Jamir and Maiti (2014) studied the effect of IBA and NAA on rooting of banana cultivars GrandNaine and Jahaji. For rooting, NAA and IBA were used individually at the concentration of 0, 0.1, 0.2 and 1 mg/l. Cent percent rooting and also highest no. of functional roots (6.33 and 5.2) with moderate root length (2-4 cm) were achieved on medium with 1 mg/l NAA. Shashikumar*et al*. (2015) found that MS medium supplemented with 1.0 mg/l IBA induced 5.33 ± 1.21 numbers of roots with a mean root length of 7.50 ± 1.87 cm in banana cv.

KaribaleMonthan. Paulos*et al*. (2015) undertook to study the effect of various concentration of growth regulators IAA (indole-3-acetic acid) (0.0, 0.5 and 1.0 mg/l) and IBA (indole-3- butyric acid) (0.0, 0.5, 1.0 and 1.5 mg/l) and their interaction on rooting in banana (*Musaparadisiaca*) cv. Grand Naine. The short duration were recorded at0.5 mg/l IAA – 8.9 days and 1.5 mg/l IBA – 9.4 days and interaction of both 0.5 mg/l IAA + 1.5 mg/l IBA has given almost a week root induction (7.33) days. The highest numbers of roots were produced by treatment of 0.5 mg/l IAA which was 6.2 and 7.8 at 15 and 30 DAI, respectively. 1.5 mg/l IBA produced 5.1 and 7.1 roots at 15 and 30 DAI respectively. In 0.5 mg/l IAA + 1.5 mg/l IBA combination gave 7.0 and 8.0 numbers of roots at 15 and 30 DAI respectively. In length of roots, it was observed that 0.5 mg/l IAA produced longest root size 5.7 and 6.7 cm at 15 and 30 DAI, respectively. The interaction, 0.5 mg/l IAA + 0.5 mg/l IBA produced 6.33 and 7.33 cm length in 15 and 30 DAI respectively. Suman and Kumar (2015) studied the effect of IBA on rooting of banana cv. Malbhog. They observed that the *in vitro* developed shoots showed 100% rooting on MS medium supplemented with 4.92 µMIndole butyric acid (IBA).

2.6. Acclimatization

Acclimatization is necessary in the case of *in vitro* produced plantlets because *in vitro* produced plant material is not adopted to natural environmental conditions (Brainerd and Fuchigami, 1981).They are very poorly adopted to resist the low humidity, higher light levels and more variable temperature prevailing outside(Wainwright, 1988). Thus, light, temperature and relative humidity are the three major factors to be controlled during acclimatization to natural environment. Physical, chemical and biological properties of potting mixture are also important factors in establishment of *in vitro* raised plantlets. Thorough washing of plantlets to remove the traces of agar and nutrient medium, dipping in 0.05% sarbendazim and sterilizing the potting mixture eliminate problem of fungal infection (Anderson, 1980 and Muniswamy*et al*., 1994). Greenhouse potting mixture used for growing out banana plantlets include 2 parts of a commercial growing media mixture (Sunshine Professional), 1 part perlite, and 3 parts vermiculite (medium to coarse grade).

Plants are generally allowed to acclimatize in the greenhouse for approximately 2 months and to reach a height of about 20 cm (8 inches) before they are transplanted to the field. (Perez and Hooks, 2008). Rai*et al*., (2012) hardened rooted plantlets of banana variety Grand Naine (G9) in

portrays containing different potting mixtures viz.soil, sand and cocopeat (1:1:1), soil sand and farmyard manure (1:1:1) and mixture of cocopeat and sand (2:1) of which, the mixture of Cocopeat and sand (2:1) showed maximum (96%) survival of plantlets. Elisama*et al*.,(2013) studied the effect of fertigation and Indole Butryic Acid (IBA) application in nutritive solution on growth of *Musa cavendischii* plantlets during acclimatization process in greenhouse. The experimental unit consisted of 1 transplanted plant and daily application of 10 ml of Steiner's nutritive solution at 10, 25, 50, 75 and 100% without and with 1mg/l of auxin (IBA).After 11 weeks of acclimatization, they found higher plants with respect to plant fresh weight, dry weight, height and leaf width which corresponded to treatment from 75 to 100% of Steiner's solution. The IBA application had no significant effects on the growth of the *M. cavendischii*plants. There was no significant interaction between fertigation and IBA applications.

Ahmed *et al.,* (2014) hardened and acclimatized *in vitro* rooted plantlets by using different treatments. Plants transplanted at the age of 4 weeks after root initiation gave maximum survival (100%) during transplanting. These plants were hardened in glass beaker and polythene bags singly or in cluster. The maximum survival during hardening (100%) was observed by covering the plantlets with glass beaker individually and kept in culture room. Out of various potting mixture tried, the potting mixture containing soil: sand and FYM (2:1:1 v/v/v) gave maximum height and survival of plantlets. The results also showed that out of different potting mixtures tried for hardening (FYM, soil, sand and vermiculite); soil: sand and plantlets in coconut coir pith to be 84.44% during primary hardening.

3. MATERIALS AND METHODS

3.1. Preparation of stock plants

For this experiment the banana cultivars used were Matoke and Nijiru. These cultivars were 3-4 months old young shoot grown in green house. The explants were obtained from healthy looking field grown suckers of these cultivars from Melkassa Agricultural Research Center (MARC) banana propagation nursery. The pseudo stems at lower parts of the suckers containing meristem s were used as explants. The shoot tips (meristem and a few leaves primordial) were the starting materials. This study was conducted at Ethiopian institute of agricultural research in the Tissue Culture Laboratory of MARC, Ethiopia.

3.2 Disinfection

For disinfection and sterilization of the mother plant 70% of alcohols for 30 second and 5% of sodium hypochlorite solution for 10 minutes were used. Then, the explants were washed three times for at least 2 minute in double distilled water. Explants were excised from young suckers (0.5 to 0.75 m) of the two banana varieties. The superfluous corm tissue, roots, and leaf sheathes were trimmed and removed from the pseudo-stem by sharp knife and finally by blade. Explants were washed thoroughly in running tap water for 15-20 minutes with detergent solution to remove adherent soils. The leaf sheaths near the bases were again removed from the pseudo-stem leaving the young leaves around the meristem until the shoot tip became about 2 cm in length.

3.3 Shoot Tip Culture Establishment

The culture medium used for this study was that of Murashige and Skoog's (1962) basal medium containing 30 g/l sucrose and gelled with 8 g/l of agar. The pH of the medium was adjusted to 5.7-5.8 using NaOH or HCl (0.1 or 1N) before autoclaving. Plant growth regulators (PGR) were also added to the medium prior to sterilization. The medium was autoclaved at 1.2 KPa and 121ºC for 20 min, and then cooled at room temperature before use. Surface sterilized explants were placed on MS medium containing a combination of N6-benzylaminopurine (BAP, 3.0 mg/l) for shoot tip initiation. The initiated cultures were incubated for 8 weeks aseptically at 25±2ºC under 16hr cool white, fluorescent lights. Evaluation of Multiplication Rate was done using the same MS medium supplemented with a combination of BAP and IAA was used for shoot

proliferation. After 8 weeks of initiation stage contamination-free shoots were decapitated at 7 to 10 mm height and split longitudinally into two or more parts depending on vigor and thickness. Then, they were transferred to multiplication medium. However, smaller shoots were not split; rather they were put in two or three in a culture vessel. The multiplication rate was studied by sub culturing the shoots on MS media every 4 weeks for 4cycles.

3.4. Media

Selection of media

Murashige and Skoog (1962) medium was selected as the basal medium throughout the experiment as it was the most favourable medium particularly for callusing and plant regeneration. MS medium is a widely accepted medium for the tissue culture of banana. For further studies an array of media was generated using MS basal medium supplemented with different combinations and concentrations of phytohormones. Besides MS basal medium, half MS medium were used.

Table 1: Composition of MS medium and stock solution

No	Stocks	Constituent	Amount(mg/l)
1	Inorganic		
	Major element	Ammonium nitrate (NH_4NO_3)	1.65
		Potassium nitrate (KNO_3)	1.90
		Calcium chloride ($CaCl_2.2H_2O$)	0.44
		Magnesium sulphate ($MgSO_4.7H_2O$)	0.37
		Potassium dihyro orthophosphate (KH_2PO_4)	0.17

2	Minor elements	Boric acid (H3BO3)	6.2
		Manganese sulphate (MnSO4.H2O)	22.3
		Zinc sulphate (ZnSO4.7H2O)	8.6
		Potassium iodide (KI)	0.83
		Sodium molybdate (Na2MoO4)	0.25
		Cupric sulphate (CuSO4.5H2O)	0.025
		Cobalt chloride (CoCl2.6H2O)	0.025
		EDTA- di sodium salt (Na2EDTA.7H2O)	37.2ml
		Ferrous sulphate (FeSO4.7H2O)	27.8ml
3	Amino acids & vitamins	Glycine (C2H5NO2)	2ml
		Inositol (C6H12O6)	1ml
		Nicotinic acid (C6H5NO2)	0.5ml
		Pyridoxine hydrochloride (C8H11NO3HCl)	0.5ml
		Thiamine hydrochloride (C12H17ClN4 O5.HCl)	0.1
4	Carbon source	Sucrose (C12H22O11)	30
5	Hormone	BAP	3
6	pH		5.7-5.8
7	Gelating agent	Agar	7-8

3.5. Culture initiation

For culture initiation, after trimming all the outer whorls of the spindle shoot tips measuring about (2-4mm) were excised and placed on MS media having BAP (3ml/l) (Murashige and Skoog, 1962). The cultures were then kept in the growth room at a temperature of 27±2^{o}C and photoperiod of 16hrs light at an intensity of 2500lux.

3.6. Shoot Multiplication

After 6-8 weeks of culture in the initiation media the prepared shoots were transferred to multiplication media for shoot formation. The medium at this stage of culture was MS basal medium added with 30 g/l of sucrose, 100mg/l of myo-Inositol, 1mg/l of thiamine, and solidified with 7g/l of agar. The media were fortified with different combination of cytokines with auxins to examine more shoot inducing treatments. These experiments were carried out by complete randomized design with eight treatments by five replications for both cultivars (Matoke and Nijiru) to evaluate at which hormone combination level plantlets show better progress.

Table 2: Treatment of shoot multiplication

Treatment(T)	BAP(ml/l)	IAA(ml/l)
T1	3	0
T2	3	0.2
T3	3	0.4
T4	3	0.6
T5	4	0
T6	4	0.2
T7	4	0.4
T8	4	0.6

Replication was five times

3.7. Rooting and Elongation

For rooting, single plantlets were transferred to MS medium supplemented with different hormone concentration levels of IAA, IBA and NAA. The experiments were conducted by complete randomized block design with 13 treatments in five replications for both cultivars (Matoke and Nijiru) to evaluate at which hormone combination level plantlets show better progress.

Table 3: Treatments of rooting

	ml/l IBA	ml/l NAA	ml/l IAA
T1	0	0	0
T2	0	0	0.5
T3	0	0	1
T4	0	0	1.5
T5	0	0	2
T6	0.5	0	0
T7	0.16	0	0
T8	0.74	0	0
T9	2.32	0	0
T10	0	0.53	0
T11	0	1.06	0
T12	0	0.59	0
T13	0	2.12	0
	Replicated five times		

3.8. Acclimatization and Hardening of Plantlet

Elongated and rooted plantlets (about 6 cm with 3 to 4 leaves) were taken out from culture vessels and roots were carefully washed thoroughly with running tap water to remove the agar. Plantlets were disinfected with Ridomyl (2.0 g/l) for 10 minutes to prevent fungal infections before transplanting. Individual plantlets were then transferred into small polyethylene bags filled with various sterile potting mixtures (sand to soils or sugarcane filter cake) in a 1:3 ratios (v/v). The potting mix was fumigated with 5% formalin ten days prior to planting (kept covered for 3 days and then aerated without cover for remaining 7 days). Plants were kept inside chambers covered with transparent plastic for a week in the green house to maintain high humidity (80 to 90%) for acclimatization and hardening. The humidity was gradually reduced and plantlets were kept outside the chamber. Then, plants were later transferred to bigger polyethylene bags that were filled with forest soil, sand and manure in the ratio of 2:1:1. The hardened plants were eventually transferred to the field and successfully established for further evaluation.

3.9. Data collected

Data on the following categories were recorded, that is completely dead, alive but not growing, or growing slowly, or growing very well and also data were record the sources of infection, as fungal or bacterial contamination. At the subsequent culture stages of shoot multiplication, as well as elongation and/or rooting data related to plantlet growth and development were recorded, as deemed necessary. These include, information on shoot and/or root development and growth, including number and length of shoots, number of leaves and length of roots.

3.10. Statistical analysis

Data collected from each experiment were subjected to statistical analyses using the SAS statistical software and ANOVA were constructed.

4. RESULTS AND DISCUSSION

4.1. Shoot Initiation

In this study, in vitro culture of banana shoot tips resulted hard meristematic ball like structure in initiation media containing 3ml BAP.The cultured shoot tip turned brown in color from the initia l creamy white in a few days after inoculation. Four weeks later, the external leaf primordia of ex plants turned green and globular hard coat mass grew from which adventitious plantlets were de eloped (Figure 1).

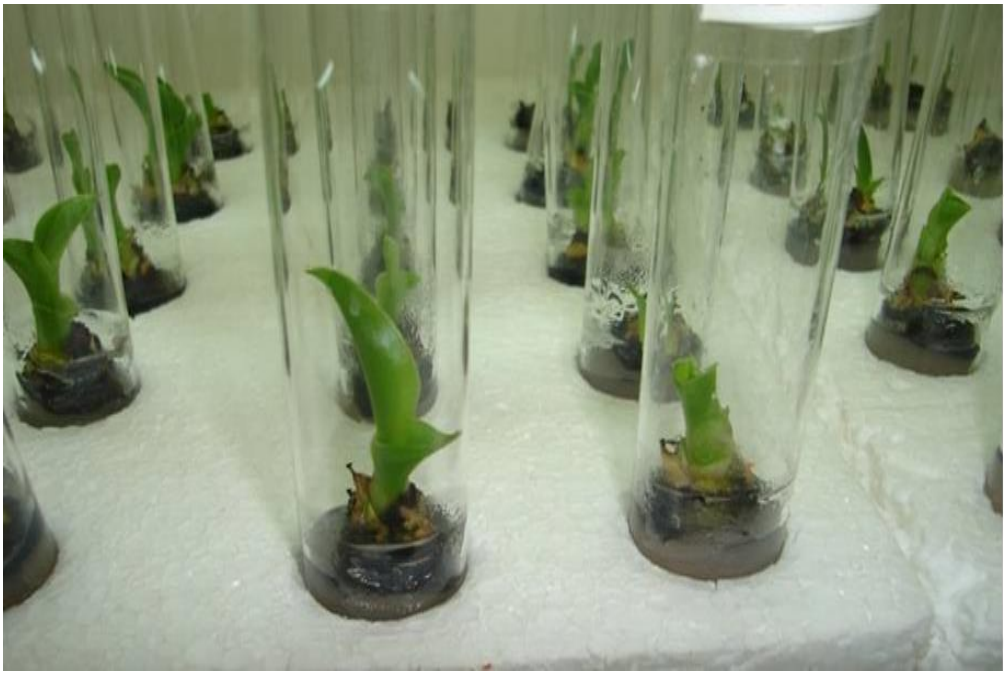

Figure 1: Shoot initiation

The data collected were depend on the percentage of survival of the cultivars. As the result indic ated, the shoot tip initiation response survival of 75% and 68% were obtained from explants cult ured on MS medium supplemented with 3.0 mg/l BAP alone for the two banana cultivars Matoke and Nijiru respectively (Table 4). According to the report of Muhammad *et al*. (2004) using BAP (2mg/l to 3mg/l) for culture imitation of banana 80% of survival were obtained. Cronauer and Krikorian (1984) and Vuylsteke (1998) also reported BAP as the most commonly preferred cytokinin used in banana tissue culture. Furthermore, Al Amin etal., (2009) observed the color

Change of culture meristems to be brown in 4 to5 days and a development of a green hard ball li ke structure after 30 to 35 days of inoculation.

Table 4: Effect of concentrations of BAP on shoot initiation of Matoke and Nijiru cultivars

Variety	Hormone concentration ml/l	% of survive
Matoke	3ml BAP	75
Nijiru	3ml BAP	68

4.2. Shoot multiplication and proliferation

4.2.1. Effect of different concentrations of BAP and IAA on shoot proliferation of Matoke and Nijiru cultivars in MS medium

In this study, the data collected depend on the number of shoot and shoot multiplication rate of the cooking banana cultivars. It was observed that the mean shoot number and multiplication rate of both cultivars were listed among the explants of the different genotypes indicated in Table 5 and 6. The results of this study were for the mean of all parameters for both cultivars were Significance difference among the treatments (P at 0.05). After 2 months of culture initiation, sli ced shoots were transferred on multiplication medium after which shoots with at least one leaf w ere emerged, (Figure2)

Figure 2 : shoot proferation Matoke and Nijiru cultivars

Vani and Reddy (1999) dissected shoot tips from healthy suckers of banana (*Musa* sp.) varieties Dwarf Cavendish, Amruthapani, Tella Chakkerakeli and Robusta, and inoculated on MS medium

supplemented with 6 mg/l BAP, 2mg/lIAA and 200 mg/l adenine sulfate. According to the report shoot initials developed after 3 weeks and were proliferated on induction medium with the BAP concentration reduced to 4 mg/l. Shoots were rooted on MS medium containing 2 mg/l BAP,2mg/l IAA and 0.1% activated charcoal. Cronauer and Krikorian, (1984) reported that multi ple shoots could be produced from sliced shoot tips of banana and plantain.

Table 5: Effect of different concentrations of BAP and IAA on shoot proliferation of Matoke cultivar in MS medium

Treatment	Concentrations of BAP and IAA	Shoot number	Multiplication factor
1	3ml BAP*0ml IAA	9.0^{bc}	8.6^{b}
2	3ml BAP*0.2ml IAA	7.0^{d}	8.0^{b}
3	3ml BAP*0.4ml IAA	8.4^{cd}	9.0^{b}
4	3ml BAP*0.6ml IAA	14.2^{a}	14.4^{a}
5	4ml BAP*0ml IAA	9.6^{bc}	7.2^{b}
6	4ml BAP*0.2ml IAA	10.0^{ab}	9.2^{b}
7	4ml BAP*0.4ml IAA	9.8^{bc}	9.0^{b}
8	4ml BAP*0.6ml IAA	10.2^{ab}	8.6^{b}
CV (%)		14.0	17.0
LSD @ 5%		1.8	2.0

Means expressed in different letters showed statistically significance difference among the treatments, LSD= Least significant difference, CV= Coefficient of variation

As the result indicated the highest number of shoot and shoot multiplication rate were obtained on MS medium supplemented with a combination of BAP and IAA at concentrations of 3mlBAP +0.6ml IAA mg/l (14.2) for Matoke cultivar and Nijiru cultivar was most productive and produce maximum number of shoots at concentration of BAP and IAA; 3ml BAP+0.2mlIAA (11.6) followed by 3ml BAP+0.4ml IAA (11.4). The poor response of shoot proferation were observed both in shoot number and multiplication rate for Matoke cultivar were at PGR concentration 3ml BAP+0.2mlIAA(7) and 4mlBAP+0IAA(7.2) respectively. The highest numbers of shoots were prod

uced on MS supplemented with 5.0mg/l BAP+1.5mg/lIAA+10% CW and 6.0 mg/l BAP + 1.5 mg/lIAA+10% coconut water (Muhammad Munir Iqbal, 2013).

On the other hand, the poor response of shoot initiation for Nijiru cultivar was observed in shoot number 4mlBAP+0mlIAA (7.4). For multiplication rate of Nijiru cultivar 3mlBAP*0.6mlIAA (7.4) and 4ml BAP*0.6mlIAA(7.6) combinations of the plant growth regulators were shown poor respon se.The mean number of shoots and multiplication rate of all treatments obtained after four subc ultures of both cultivars were stated table 5and 6. The result showed that there were significance differences among treatments on both cultivars in all parameters. In this experiment, sub culturing of the shoots for multiplication was carried out for 4 cycles. It was observed that the cul tures showed higher rate of multiplication for the first four subcultures. According to the report of Muhammad Munir Iqbal, 2013 the best results clearly showed that 5.0 BAP+1.0 IAA+10%C W media most productive for shoot proliferation.

However, after the 4th cycle the multiplication rate was declined. Sub culturing induced multiple axillary shoots were more than a three-fold increase in multiplication was seen by three weeks after the first subculture. Further transfer in the same medium resulted in about 2 to 4 fold increase in proliferation at every subculture cycle. The meristematic shoot tip explants were inoculated on MS medium with eight different combinations of BAP and IAA. Among the various treatments, the effective results were obtained from these eight combinations given in the Table 5and 6.

Table 6: Effect of different concentrations of BAP and IAA on shoot proliferation and multiplication of Nijiru cultivar in MS medium

Treatment	Concentrations of BAP and IAA	Shoot number	Multiplication factor
1	3ml BAP*0ml IAA	12.2[a]	15.0[a]
2	3ml BAP*0.2ml IAA	11.6[a]	12.4[b]
3	3ml BAP*0.4ml IAA	11.4[a]	11.8[b]
4	3ml BAP*0.6ml IAA	8.4[b]	7.6[c]
5	4ml BAP*0ml IAA	7.4[b]	7.8[c]
6	4ml BAP*0.2ml IAA	8.0[b]	9.4[c]
7	4ml BAP*0.4ml IAA	8.8[b]	9.4[c]
8	4ml BAP*0.6ml IAA	8.2[b]	7.6[c]
CV (%)		17.4	17.7
LSD @ 5 %		2.1	2.3

Means expressed in different letters manuscript showed statistically significance difference among treatments, LSD= Least significant difference, CV= Coefficient of variation

After 2-3 weeks, explants changed colour to green and produced shoots.The mean number of sho ots and multiplication rate of all treatments obtained after four subcultures were stated in table 5 and 6.The results showed that 75% contamination free cultures were obtained. When these antibiotic treated explants were cultured in MS, they produced healthy shoots (Figure 2). After four sub-culturing, the clump formation occurs and the capability of culture to further divide gradually becomes low. The proliferating axillary buds were well defined, pale green and 3.0-4.0 cm long with bulbous base and thin pointed tips. After 3-4 weeks, threefold increase in multiplication was seen. At every subculture in the same media resulted in three to four fold increases in multiplicate axillary buds Proliferating shoots continue to produce axillary buds on the same fresh media while elongated shoots were transferred to root induction media for root de velopment.

According to Kalimuthu *etal.,* (2007) among thirteen different combinations with various concentrations of BAP and IAA were used in their study to analyze the shoot initiation and shoot multiplication and resultantly MS medium of 5.0mg/l of BAP and 1.0mg/l of IAA+10% coconut

water showed good results both for shoot initiation and multiplication. Auxins and cytokinins ha ve been reported as the most frequently used plant growth regulators for banana micropropagatio n (Vuylsteke, 1998). Adenine-based cytokinin particularly BAP is the most commonly preferred cytokinin to affect shoot multiplication rate in several *Musa* spp. (Cronauer and Krikorian, 1984; Vuylsteke, 1998). Differences in rate of multiplication of different *Musa* genotypes under in vitro conditions have also been reported (Vuylsteke, 1998; Muhammadi*et al*., 2004). In the present investigation, shoot meristems were cultured on agar (semisolid) similar reports were also made by Kalimuthu *et al.,* (2007).

4.3. Rooting and Elongation

4.3.1. Effect of different concentrations of IBA, NAA and IAA on explant of Matoke and Nijiru cultivars rooting in MS medium

In this study the data were collected for rooting and elongation depend on four parameters. Those parameters were: shoot number, shoot length, root number and root length of the plantain (Matoke and Nijiru cultivars). The result of this study were for the mean of all parameters for both cultivars were Significance difference among the all treatments (P at 0.05).Three different auxins (IAA, NAA, and IBA) were used in three different combinations in MS supplemented medium for root induction. The best progresses were obtained at PGR concentration of IBA 0+NAA 0.59+ IAA 0 on all parameters (shoot number (3.8), shoot length (18.7cm), root number (17) and root length (21.2cm)) for Matoke cultivar. But, the poor progresses were obtained at different PGR concentration for Matoke cultivar on those parameters. That were: the poor shoot number was obtained at hormone concentration 0.5IBAml/l (0.6), control and 0.16ml/l IBA (1): for shoot length 0.7cm at concentration of 0.5 IBA and 1.8cm at control treatment: for number roots 0.2 at concentration of 0.5IBAml/l, 1.4 at concentration of 0.74IBAml/l and 1.8 on control treatment: and for root length at PGR concentration of 0.5IBAml/l 0.3cm and at 0.16IBAml/l 0.8cm (Table 7).

Table 7: Effect of different concentrations of IBA, NAA and IAA on explant of Matoke cultivar rooting in MS medium

Treatment	concentrations of IBA, NAA and IAA(ml/l)	SHPP	SHL(cm)	RPP	RL(cm)
1	IBA 0* NAA 0,*IAA 0	1.0^{ef}	1.8^{fg}	1.8^{ef}	5.2^{f}
2	IBA 0*NAA 0*IAA 0.5	1.8^{cde}	2.78^{f}	3.6^{e}	6.9^{e}
3	IBA 0* NAA 0*IAA 1	1.0^{ef}	6.1^{e}	7.2^{d}	4.4^{f}
4	IBA 0*NAA 0* IAA 1.5	2.6^{bc}	6.7^{de}	10.6^{c}	2.7^{g}
5	IBA 0*NAA 0*IAA 2	1.6^{de}	5.9^{e}	7.0^{d}	7.3^{e}
6	IBA 0.5*NAA 0*IAA 0	0.6^{f}	0.7^{g}	0.2^{f}	0.3^{h}
7	IBA 0.16*NAA 0*IAA 0	1.0^{ef}	7.8^{dc}	14^{b}	0.8^{h}
8	IBA 0.74*NAA 0* IAA 0	3.0^{ab}	5.8^{e}	1.4^{f}	6.6^{e}
9	IBA 2.32*NAA 0* IAA 0	1.6^{de}	8.8^{c}	14.0^{b}	9.3^{d}
10	IBA 0* NAA 0.53* IAA 0	1.8^{de}	12.78^{b}	17.0^{a}	13.6^{c}
11	IBA 0* NAA 1.06* IAA 0	1.2^{ef}	13.54^{b}	14.6^{b}	18.8^{b}
12	IBA 0*NAA 0.59* IAA 0	3.8^{a}	18.7^{a}	17.0^{a}	21.2^{a}
13	IBA 0* NAA 2.12*IAA 0	2.4^{bcd}	13.2^{b}	10.0^{c}	14.4^{c}
CV (%)		18.9	11.8	18.9	11.5
LSD @ 5%		0.8	1.2	2.0	1.3

Means expressed in different letters manuscript showed statistically significant different among treatments, LSD= Least significant difference, CV= Coefficient of variation

The best number of shoot for Nijiru cultivar was at PGR concentration IBA 0.74+NAA 0+ IAA0 (4) and IBA 2.32+NAA0+IAA0 (3.6). In other case, the poor number of shoot were observed at hormone concentration IBA0+NAA2.12+IAA0 (0.8) followed by IBA 0+NAA 0+IAA 0.5(1) and IBA 0+ NAA 0.53+IAA0 (1) for Nijiru cultivar. As the result indicated the best shoot length was observed at hormone concentration IBA 0.74*NAA 0* IAA 0 (12.7cm) and the poor one were at hormone concentration IBA 0+NAA 0.59+ IAA 0 (1.2cm) and IBA 0+NAA 2.12+IAA 0 (1.06cm) for Nijiru cultivar.

According to this study the best progress of root number was obtained at hormone concentration of IBA 0.74+NAA 0+ IAA0(14.4) and the poor root number was obtained at hormone concentration of IBA 0+NAA 2.12+IAA0 (2.8) for Nijiru cultivar and the good progress of root length was obtained at hormone concentration of IBA 0.74+NAA 0+IAA0 (13.6cm) and the poor plant root length were obtained at hormone concentration of IBA0+NAA 2.12+IAA0 (1.88) and IBA0+NAA0+IAA1 (2.7cm) for Nijiru cultivar(Table 8).

According to the report of Muhammad Munir Iqbal, 2013 the results showed that MS medium supplemented with IAA (2.0mg/l) gave best results which produced 60% root induction.Four diff erent auxins (ABA, NAA, IBA and IAA) with varying concentrations were employed to check their effect on root induction. Each auxin was applied in four different concentrations (0, 0.5, 1.0 and 2.0mg/l). For root induction, the well grown shoots were separated and transferred to MS media containing varying concentrations of auxins (Muhammad Munir Iqbal, 2013).

According to the experiment effect of different concentrations of IBA, NAA and IAA on explant of Matoke cultivar rooting in MS medium were studied with thirteen treatments with five replication. The mean number of shoot, shoot length, root number and root length for Matoke and Nijiru cultivars across the treatment were listed in Table 7 and 8 respectively.

Table 8: Effect of different concentrations of IBA, NAA and IAA on explant of Nijiru cultivar rooting in MS medium

Treatment	Concentrations of IBA,NAA and IAA(ml/l)	SHPP	SHL(cm)	RPP	RL(cm)
1	IBA 0* NAA 0,*IAA 0	1.6^{cd}	3.8^{e}	5.6^{e}	4.6^{e}
2	IBA 0*NAA 0*IAA 0.5	1.0^{cd}	3.5^{e}	5.2^{e}	4.4^{ef}
3	IBA 0* NAA 0*IAA 1	1.2^{cd}	1.86^{fgh}	4.0^{fghi}	2.7^{fgh}
4	IBA 0*NAA 0* IAA 1.5	1.0^{cd}	2.6^{ef}	5.0^{egh}	3.7^{efg}
5	IBA 0*NAA 0*IAA 2	1.8^{bc}	7.7^{c}	9.6^{c}	8.7^{c}
6	IBA 0.5*NAA 0*IAA 0	2.6^{b}	8.74^{bc}	10.8^{bc}	9.76^{bc}
7	IBA 0.16*NAA 0*IAA 0	1.8^{bc}	5.1^{c}	7.2^{d}	6.1^{d}
8	IBA 0.74*NAA 0* IAA 0	4.0^{a}	12.7^{a}	14.4^{a}	13.6^{a}
9	IBA 2.32*NAA 0* IAA 0	3.6^{a}	9.2^{b}	11.0^{b}	10.2^{b}
10	IBA 0* NAA 0.53* IAA 0	1.0^{cd}	2.3^{fg}	4.2^{fgh}	3.3^{fgh}
11	IBA 0* NAA 1.06* IAA 0	1.6^{cd}	1.7^{fgh}	3.8^{ghi}	2.7^{ghi}
12	IBA 0*NAA 0.59* IAA 0	1.6^{cd}	1.2^{gh}	3.4^{hi}	2.2^{hi}
13	IBA 0* NAA 2.12*IAA 0	0.8^{d}	1.1^{h}	2.8^{i}	1.9^{i}
CV (%)		16.8	18.6	14.8	16.2
LSD @ 5%		0.8	1.1	1.2	1.2

Means expressed in different superscript letters showed statistically significance difference among treatments, LSD= Least significant difference, CV= Coefficient of variation

According to Cronauer and Krikorian (Cronauer andKrikorian,1984) Naphthalene acetic acid (NAA) was found to be effective at very low concentrations for root initiation of banana. NAA 1.0 mg/l is suitable for root initiation in *Musa sp*. Arinaitwe et al.(2000) observed rooting on MS medium containing NAA 1.2 µM during the study of multiplication rate effects of cytokinins on Kibuzi, Bwara and Ndizwemit banana cultivars. Pruski et al.(2005) also reported best rooting response in a combination of IBA and NAA. However, De Langhe (1985) and Novak et al. (1990) used half strength MS+1.0mg/l IBA, whereas Cronauer and Krikorian(1984) used auxin free MS for

rooting of banana microshoots. On the other hand, Banerjee and De Lahange (1986) and Azad a nd Amin (2001) obtained rooted banana shoots in half strength MS medium supplemented with 0 .2mg/lIBA.Akbar and Roy (2006) reported 1.0 mg/l IBA for best rooting response of *in vitro* cultured plants. Rooting can be stimulated when individual shoots are transferred to a basal medium without any PGR (Cronauer and Krikorian, 1984; Jarret*et al*., 1985). However, auxins induce further root initiation in bananas (Vuylsteke, 1989).

A similar result was reported by Vessey and Rivera (1981) that the root formation was occurred 50 days after shoot transfer. However, Berg and Bustamante (1974) noted that plantlets needed 2 to 3 months for root formation. Synthetic growth regulating chemicals that have been found most reliable in stimulating adventitious root production are the auxins, that is, IAA, NAA, IBA and 2,4- D (Arteca, 1996). Auxins, (IBA, IAA, and NAA at concentration of 0.2, 1.0 and 0.5 mg/l respectively) are most frequently used to induce root initiation in the banana (Vuylsteke, 1989; Arteca, 1996). During the current study, the root induction and elongation response to IAA were better than for all other auxins evaluated. These findings are contrary to those of Vuylsteke (1989) who reported that NAA was more effective than IAA in banana tissue culture.

Figure 3: Matoke and Nijiru cultivars on rooting stage

4.4. Acclimatization of In Vitro Rooted Plants

The well developed and healthy in vitro raised plantlets (about 6cm with 3-4 leaves) were transferred into small plate bed filled with different soil media mix, and kept inside small chambers covered with transparent plastic in the greenhouse for primary hardening for a week (Figure 4). Plantlets were washed by distilled water and disinfected with Ridomyl (1.0 g/l) for 10 minutes to prevent fungal infections before transplanting. Individual plantlets were then transferred into soil filled plate bed .Thorough washing of plantlets to remove the traces of agar and nutrient medium and sterilizing the potting mixture eliminate problem of fungal infection (Anderson, 1980 and Muniswamy *et al*., 1994). Plantlets were then transferred to plate bed filled with soil media for acclimatization. All the plants were acclimatized (85% survival), and the high est vegetative plant growth in terms of plant height, pseudo stem girth and number of photosynth etic leaf per plant was recorded on media containing sugarcane filter cake and sand at 3:1 ratio (v/v). The survival plantlets were planted in soil filled polyethylene bag. The hardened plants were transferred to the field, successfully established, and produced large bunches with high quality fruits

Figure 4: Root plantlets being acclimatized

5. CONCLUSIOIN AND RECOMMENDATION

This study was aimed to develop a simple, comprehensive and efficiently repetitive protocol for micropropagation of banana (*Musa sapientum* L.*)* using shoot meristem. The shoot tip initiat on response with survival of75% and 68% were obtained from explants cultured on MS medium supplemented with 3.0 mg/l BAP alone for the two banana cultivars Matoke and Nijiru respectiv ely. After growing in-vitro cultures on different hormonal combinations, Murashige and Skoog's medium supplemented with BAP and IAA, the highest number of shoot and shoot multi plication rate was obtained on MS medium supplemented with a combination of BAP and IAA at concentrations of 3mlBAP+0.6ml IAA mg/l (14.2) for Matoke and Nijiru cultivar were most prod uctive and produced maximum number of shoots at concentration of BAP and IAA; 3ml BAP+0.2mlIAA (11.6) followed by 3ml BAP+0.4ml IAA. Antibiotic (cefotaxime) was used to check the endogenously born bacterial contamination. Initiation of shoot bud and establishment of culture from shoot meristem was achieved on sem-solid media.

The proliferated shoots were excised and transferred to different root induction media, which resultantly showed that MS media supplemented with IAA NAA and IBA, were the most efficient root inducing media. The best progress were obtained at hormone concentration of IBA 0*NAA 0.59* IAA 0 on all parameters (shoot number, shoot length, root number and root length) for Matoke cultivar and the best number of shoot of Nijiru cultivar was at hormone concentration IBA 0.74 (4) and IBA 2.32 (3.6) respectively. In other case, the poor number of shoot were observed at hormone concentration NAA 2.12 (0.8) followed IAA 0.5(1) and NAA 0.53 (1) for Nijiru cultivar. As the result indicated the best shoot length was observed at hormone concentration IBA 0.74 (12.7cm) and the poor one were at hormone concentration NAA0.59 (1.2 cm) and NAA 2.12 (1.06cm).According to this study the best progress of root number was obtained at hormone concentration of IBA 0.74 (14.4).Rooted plantlets after primary and second ary hardening were transferred to the green house. Finally, these disease free plants were success fully established in soil and distributed for end user.

Generally, I recommend;

- For initiation of both Matoke and Nijiru cultivars PGR of 3mlBAP were better progress.
- For shoot and multiplication at plant growth regulators 3mlBAP+0.6ml IAA mg/ and 3ml BAP+0.2mlIAA better for Matoke and Nijiru cultivars respectively.

- For rooting and elongation at plant growth regulators of 0.59NAA and 0.74 IBA were better progress for Matoke and Nijiru cultivars rooting induction respectively.

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7. APPENDICES

Table: ANOVA of number of shoot Matoke cultivar (multiplication)

Source	DF	Squares	Mean Square	F Value	Pr > F
Model	7	150.1750000	21.4535714	11.29	<.0001
Error	32	60.8000000	1.9000000		

Table: ANOVA of multiplication factor Matoke cultivar (multiplication)

Source	DF	Squares	Mean Square	F Value	Pr > F
Model	7	166.3000000	23.7571429	9.60	<.0001
Error	32	79.2000000	2.4750000		

Table: ANOVA of number of shoot Nijiru cultivar (multiplication)

Source	DF	Squares	Mean Square	F Value	Pr > F
Model	7	126.8000000	18.1142857	6.65	<.0001
Error	32	87.2000000	2.7250000		

Table: ANOVA of multiplication factor Nijiru cultivar (multiplication)

Source	DF	Squares	Mean Square	F Value	Pr > F
Model	7	254.7750000	36.3964286	11.24	<.0001
Error	32	103.6000000	3.2375000		

Table: ANOVA of shoot number of for Matoke cultivar (rooting)

Source	DF	Squares	Mean Square	F Value	Pr > F
Model	12	51.20000000	4.26666667	10.47	<.0001
Error	52	21.20000000	0.40769231		

Table: ANOVA of shoot length for Matoke cultivar (rooting)

Source	DF	Squares	Mean Square	F Value	Pr > F
Model	12	1646.253538	137.187795	151.56	<.0001
Error	52	47.068000	0.905154		

Table: ANOVA of root number for Matoke cultivar (rooting)

Source	DF	Squares	Mean Square	F Value	Pr > F
Model	12	2064.738462	172.061538	66.18	<.0001
Error	52	135.200000	2.600000		

Table: ANOVA of root length for Matoke cultivar (rooting)

Source	DF	Squares	Mean Square	F Value	Pr > F
Model	12	2620.577846	218.381487	223.89	<.0001
Error	52	50.720000	0.975385		

Table: ANOVA of shoot number of for Nijiru cultivar (rooting)

Source	DF	Squares	Mean Square	F Value	Pr > F
Model	12	61.20000000	5.10000000	12.51	<.0001
Error	52	21.20000000	0.40769231		

Table: ANOVA of shoot length for Nijiru cultivar (rooting)

Source	DF	Squares	Mean Square	F Value	Pr > F
Model	12	819.4633846	68.2886154	87.43	<.0001
Error	52	40.6160000	0.7810769		

Table: ANOVA of root number for Nijiru cultivar (rooting)

Source	DF	Squares	Mean Square	F Value	Pr > F
Model	12	788.2461538	65.6871795	66.20	<.0001
Error	52	51.6000000	0.9923077		

Table: ANOVA of root length for Nijiru cultivar (rooting)

Source	DF	Squares	Mean Square	F Value	Pr > F
Model	12	828.2541538	69.0211795	81.62	<.0001
Error	52	43.9760000	0.8456923		


PEEL OFF SHIPPING TAPE
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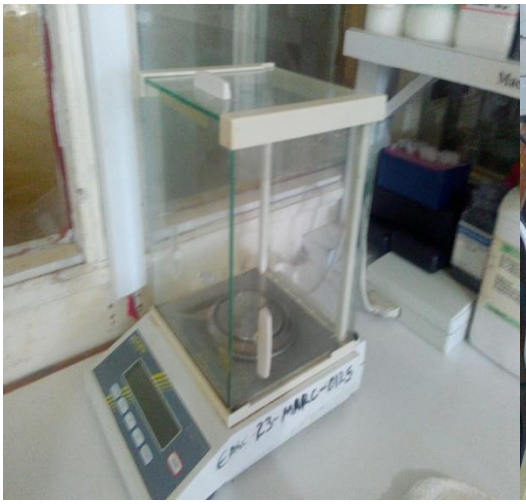
EML 23-MAR-2015


stuart