IN VITRO MICROPROPAGATION OF BANANA

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CERTIFICATE

This is to certify that thesis entitled, "*IN VITRO* MICROPROPAGATION OF BANANA" submitted to the Faculty of Agriculture, Sher-e-Bangla Agricultural University, Dhaka, in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE in HORTICULTURE AND POSTHARVEST TECHNOLOGY embodies the result of a piece of *bona fide* research work carried out by Md. Rezaul Karim, Roll No. 60, Registration No. 23968/00202 under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.

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

.....

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Dated:
Place: Joydebpur, Gazipur.

DEDICATED TO

MY

BELOVED PARENTS

ABBREVIATIONS AND ACRONYMS

BAP	:	6- Benzyl Amino Purine
BA	:	Benzyladenine
BARI	:	Bangladesh Agricultural Research Institute
Cm	:	Centimeter
CRD	:	Completely Randomized Design
cv.	:	Cultivar
DAI	:	Days after inoculation
Dw	:	Distilled water
g/l	:	Gram per litre
IAA	:	Indole acetic acid
IBA	:	Indole buteric acid
NAA	:	α- napthalene acetic acid
$Kgcm^{-2}$	:	Kilogram per sq. centimeter
LSD	:	Least Significant Difference
mgL^{-1}	:	Milligram per litre
MS	:	Murashige and skoog
PGR	:	Plant Growth Regulators

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ABSTRACT

The present study was conducted at the Biotechnology Laboratory, Biotechnology Division, Bangladesh Agricultural Research Institute (BARI), Gazipur during the period from September, 2004 to June, 2005 to investigate the effect of different concentrations of BAP and NAA on virus free plant regeneration, shoot multiplication and different concentrations of IBA and IAA on *in vitro* root formation of banana cv. BARI-1.

The culture meristem first turned brown in colour in 4-5 days and after 30-50 days later a green globular hard coat mass grow to be round in shape producing a ball like structure. From this ball like structure adventitious plantlets were developing.

Among the different concentration 7.5 mgL^{-1} BAP + 0.5 mgL^{-1} NAA showed the highest shoot proliferation 0.75, 2.75 and 6.25 shoots per explant at 10, 20 and 30 DAI respectively. The longest shoot was produced by the treatment concentration 7.5 mgL^{-1} BAP + 0.5 mgL^{-1} NAA treatment (1.03, 2.45 and 3.38 cm) at 10, 20 and 30 DAI respectively. The maximum number of leaves (2.50, 3.25 and 7.00 leaves/explant at 10, 20 and 30 DAI) produced on the medium supplemented with 7.5 mgL^{-1} BAP and 0.50 mgL^{-1} NAA. The longest leaves was produced by the treatment concentration 7.5 mgL^{-1} BAP + 0.5 mgL^{-1} NAA treatment (0.85, 2.70 and 4.23 cm at 10, 20 and 30 DAI) respectively. For root initiation half strength MS medium supplemented with different levels of IBA (0, 0.5, 1.0 and 1.50 mgL^{-1}) and IAA (0, 0.5 and 1.0 mgL^{-1}) in order to allow root formation. Root numbers varied with different concentrations of IBA and IAA. The highest number of roots were produced by 0.5 mgL^{-1} IAA + 0.5 mgL^{-1} IBA (3.50, 4.50 and 6.50 per explant respectively). The highest length was observed at 10, 20 and 30 DAI in the treatment concentration 0.5 mgL^{-1} IAA and IBA (2.93, 4.63 and 5.88 cm) which was statistically significant. Meristem derived plantlet transferred to polybags containing 1:1 (ground soil : cowdung) mixture after 7 days hardening in room temperature (28-30°C) and established plantlet is ready for planting.

CHAPTER I

INTRODUCTION

The banana and plantains (*Musae spp.*) belonging to the family *Musacae* are one the world's most important subsistence crops. It is originated in Malaysia through a complex hybridization process (Novak, 1992). It is widely grown in the tropics and subtropics in all types of agricultural system, from small, mixed, subsistence gardens, to large commercial monocultures. The crop serves in many developing countries as a staple food or the cornerstone of the country's economy. The largest producers are Latin America and Asia, however, much of the South American production is as an export crop to the developed world.

In Bangladesh, banana is popular for its year round availability, abundant production as well as high acceptability to the consumers. It is a rich source of carbohydrate and also rich in some minerals, notably phosphorus, calcium and potassium. Banana is particularly rich in vitamin-C and also contains significant amounts of several other vitamins (INIBAP, 1987). In addition, it has importance for tannin, latex and fiber production.

Banana ranks first in terms of production and second in terms of area, among the fruit crops and so has commercial value in Bangladesh. It comprises nearly 42% of the total fruit production of the country. It occupies an area of 43 thousand hectares of land with total production of 606 thousand metric tonnes with an average yield of 14.16 t/ha (BSS, 2003). This yield is quite low compared to other banana growing countries of the world like Argentina (34 t/ha) and Costa Rica (33t/ha) (FAO, 2002).

Banana is also the premier fruit of Asia and the Pacific. It is the most important fruit of Indonesia, Thailand, Bangladesh, Vietnam, the Philippines, the South Pacific island countries and also India, where recently banana has been surpassing mango, traditionally the dominant fruit. Banana also occupies an important position in the agricultural economics of Australia, Malaysia, Taiwan Srilanka and South China. Taiwan and the Philippines derive substantial earnings form their banana export industries the great bulk of bananas produced in our region are traded and consumed in domestic markets.

The low yield and production of banana is influenced by many natural and field factors, problem of virus free planting materials being the major among them. The traditional clonal propagation method appears unable to satisfy the increase in demand for disease free and healthy planting materials of banana. The productivity of vegetative propagated banana crops, such as banana and plantain is greatly reduced by virus disease (Lepoivre, 2000).

To minimize the above mentioned problems, micropropagation could be an alternative for propagation of planting materials for banana. In this method, over a million of plant can be grown from a small or even a microscopic piece of plant tissue within a year (Mantell *et al.*, 1985). Moreover the shoot multiplication cycle is very short (2-6 weeks), each cycle resulting in an exponential increase in the number of shoots and plant multiplication can be continued through out the year irrespective of the season (Razdan, 1993).

Meristem culture offers an efficient method for rapid clonal propagation, production of virus free materials and germplasm preservation in plants (Cronauer and Krikorian, 1984a; Hwang *et al.*, 2000 and Helloit *et al.*, 2002). Moreover, the

shoot proliferation cycle is very short (2-6 weeks) and each cycle resulting in an exponential increase in number of shoots and the plant proliferation can be continued irrespective of the season (Razdan, 1993). But conventionally, 5-10 suckers can be obtained per plant per year which may not be of uniform size and virus free planting materials.

As regards yield performance, tissue cultured plants have been reported to produce 39% higher yield than plants from sword suckers (Pradeep *et al.*, 1992). Under Bangladesh conditions, tissue culture derived plantlets of banana performed better than the conventional sword suckers (Faisal *et al.*, 1998).

Plant growth regulators are the essential part for *in vitro* regeneration of crop plants grown in any artificial medium. Generally, cytokinin helps in shoot proliferation and auxins helps in rooting of proliferated shoots. However, the requirement of cytokinin and auxins depends on the variety of banana and culture conditions (Cronauer and Krikorian, 1984a). BARI-1 banana variety plays a vital role in our national economy due to their popularity and acceptability to marginal and commercial farmers. To obtain virus and disease free healthy planting materials, development of a protocol for meristem culture of banana cv. BARI-1 are of prime importance. Therefore, considering the above facts the present study was undertaken with the following objectives:

i) To study the effect of BAP and NAA growth regulators on *in vitro* meristem culture (virus free) and shoot proliferation of banana cv. BARI-1

ii) To determine the effect of IBA and IAA growth regulators and their concentration required for banana *in vitro* root development

iii) To develop protocol for *in vitro* rapid propagation of banana

CHAPTER II

REVIEW OF LITERATURE

Banana and plantain (*Musa spp.*) are the quick growing fruit crops in Bangladesh. Bananas are generally propagated by suckers. But now-a-days they are also propagated through tissue culture. Generally, micropropagation of plants through *in vitro* meristem culture is done to obtain healthy, disease free plants or suckers. *In vitro* culture depends on various factors, like composition of media, explants and environmental conditions e.g. temperature, light, humidity etc. Some works have been done on these aspects in many countries of the world, whereas it is very limited in Bangladesh. In this chapter different works on *in vitro* meristem culture, elimination of banana viruses by meristem culture, plantlets production and their adaptation in the rooting media have been reviewed.

2.1 Shoot induction of banana

Ganga and Chezhiyan (2002) carried out an investigation on *in vitro* cultures of banana where four diploid cultivars, Sannachenkadali (AA), Anaikomban (AA), Kunnan (AB) and Thattillakunnan (AB) were treated with two antimitotic agents, colchicine ($C_{22}H_{25}NO_6$) and oryzalin (3,5-dinitro-N_4, N_4-dipropylsulfanilamide) to induce ploidy alterations, particularly the induction of tetraploids. They found that both antimitotic agents, but particularly colchicines, had a negative effect on the *in vitro* regeneration of the four cultivars. This was reflected in terms of the delay in regeneration, reduced multiple shoot regeneration rates, regeneration of smaller micro shoots with lower fresh weights, and reduced response to rhizogenesis. Colchicine, as expected, had a negative effect on the number of multiple shoots regenerated. However, oryzalin at lower concentrations (10 and 20 μM) resulted in the

regeneration of more micro shoots per culture than the untreated control. The chromosome doubling capacity of colchicine was equal to that of oryzalin only at 125-200 times' higher concentrations.

Helliot et al (2002) carried out an experiment on the utilization of cryopreservation for the eradication of cucumber mosaic virus (CMV) or Banana Streak Virus (BSV) from *Musa* spp. The health status of regenerated *in vitro* plants was checked by means of ELISA. The results showed that the frequency of virus eradication for CMV and BSV was 30% and 90%, respectively following cryopreservation. In comparison, the frequency of virus free plants regenerated directly from highly proliferating meristem, corresponding to a spontaneous eradication rate, reached 0% and 52% for CMV and BSV, respectively. The conventional meristem culture resulted in 0% CMV free plants and 76% BSV free plants, while the cryoprotective treatment resulted in 2% CMV free plants and 87% BSV free plants. To understand the mode of action of cryopreservation for the eradication of viral particles, they examined the structure of the meristem tips by light microscopy. The cryopreservation method used only allowed survival of small areas of cells located in the meristematic dome and at the base of the primordia.

Habiba et al (2002) reported that the best medium for single shoot development to obtain contamination free culture of the table bananas *Musa sapientum* cv. Chini Champa and Amritasagar was MS media supplemented with 4.0 mgL^{-1} BAP and 1.0 mgL^{-1} kinetin. Whereas the best medium for shoot multiplication was MS medium fortified with 4.0 mgL^{-1} BAP, 2.0 mgL^{-1} IAA and 13% coconut water. Average time required for production of single shoot and multiple shoot were 15-21 days and 40-45 days, respectively.

Sa *et al.* (2002) outlined the technique of *in vitro* propagation of banana using material from Minas Gerais, Brazil. The results were summarized of the effect of medium supplemented with benzylaminopurine (benzyladenine), sucrose and agar at pH 5.7 on establishment of explants.

The *in vitro* techniques were developed by Helliot *et al.* (2001) to eliminate of virus diseases from *Musa sp.* banana cv Williams BSJ (ITCO579) Plants were infected by one of the 3 following viruses: Cucumber mosaic virus, banana bunchy top virus and banana streak virus. Meristems were excised from *in vivo* plants, from *in vitro* plants and from highly proliferating meristems. The results showed that eradication rates varied according firstly to the type of virus and secondly to the plant materials from which the meristem was excised.

From an investigation on Fusarium Research and Development in Taiwan, Hwang *et al.* (2000) reported that meristem culture technique was very useful in stopping the spread of the pathogen from infected plantations to disease free plantations via infected suckers.

Sterilized meristem (1, 2, 3, 4 and 5 mm in size) of banana plantlets infected with BBTV or BMV (cvs. Grand Nain, Williams and Dwarf) were cultured on MS medium supplemented with 3 mg/l BAP [benzyladenine] and incubated under control conditions and plantlets were tested by DAS-ELISA for the presence of the viruses (Allam *et al.*, 2000). Obtained data indicated that virus eradication depended on the size of the excised meristem, Virus type and cultivars. The showed that when rhizomes infected with BBTV and BMV were ox posed to wet heat (50-75°C) for 60 min and dry heat (35-75°C) for 24 hrs and cultured in sterilized soil. Data illustrated that at no temperature of wet on dry heat treatment did any of the rhizomes become

free from BBTV. However, 50, 55, 60 and 65°C for 24 hrs cured 6.7-50 of the treated rhizomes. They studied on another experiment that rhizomes infected with BBTV or BMV were incubated at 40°C for 1,2 and 3 months and isolated meristem were cultured on MS medium and BAP was added to the basal medium indifferent concentrations (0- 5.0 mgL^{-1}) to study the effect at BAP on BBTV and BMV eradication. The results pointed out that no BBTV free plantlets were obtained in all concentrations and BMV free plantlets were obtained at 4.0 and 5.0 mgL^{1} BAP.

Silva *et al.* (1998) cultured leaf sheath disks of Nanicao banana (*Musa* sp., AAA group, Cavendish sub group) on a Murashige and Skoog (MS) basal medium supplimented with activated charcoal (0.2%) MES (2 [N- morpholino] ethane sulphonic acid) (15.3 μM) arginine (300 mμ), Picloram (414 μM) and zip [Isopen tenyladenine] (429 μM). The results also pointed out when shoots with roots were obtained from the embryo like structures and plantlets developed. The regeneration protocol presented may be useful to banana breeding via somaclonal variation.

Das *et al.* (1997) cultured shoot tip sections of the four banana cultivars Martaman, Kanchkala, Giant Governor and Singapuri on modified MS medium supplemented with NAA 0.5 mgL^{-1} and BAP 0.5 mgL^{-1}. In general, Kanchkala (*Musa*, ABB group) showed the best explants survival as well as growth response. Martaman (*Musa*, ABB group) also showed good growth but comparatively poor explants survival. Cultivar Singapuri and Giant Governor, both belonging to the Dwarf Cavendish (*Musa*, ABB group) showed a moderate response to the micropropagation technique.

Pancholi *et al.* (1996) studied the stability of meristem culture in context with somaclonal variation of banana. The results showed that 17% of plants were found to be variants and the variation was genotype dependent and was strongly influenced by the type of medium. Banana plants cultured in liquid medium showed more variation than plants cultured on solid medium.

Ganapathy et al. (1995) described a simple low cost method for micropropagation of banana cv. Basrai where commercial grade sugar and tap water can be substituted for sucrose and distilled water.

Devi and Nayar (1993) carried out an experiment by using shoot tips from 1 and 3 month old suckers of *Musa paradassica* cv. Nendran in liquid MS medium supplemented with 5.55 µM myoinositol, 2.97 µM thiamin HCl, 22.0 µM benzyladenine, sucrose and coconut water 15%. After 2-3 weeks they observed that one month old suckers produced 7-8 shoots/explant compared to 9-16 explants from 3 months old suckers.

Bhaskar *et al.* (1993) studied the effect of three types of explant viz. shoot tip, eye bud and floral apex of banana cv. Red Banana culturing on semisolid MS medium supplemented with various growth regulators. For all the three types of explant, the shortest time taken for culture establishment was obtained with 0.5 ppm. NAA+ 3.00 ppm. BA. GA had a negative effect on culture establishment. The greatest number of shoot (9-12 per explant) was obtained on medium supplemented with 10.0 mgL^{-1} BA regardless of NAA concentration.

An *in vitro* method was developed by Seeni and Latha (1992) to regenerate large numbers of phenotypically uniform plants from the basal parts of the leaves of

flowering plants. Differentiations of up to 10 shoot buds free of callus and protocorm like bodies occurred in 10-12 weeks from the base of a single leaf implanted in Mitra medium supplemented with 2% sucrose, 2.0 gL^{-1} peptone, 44.4 µM BA and 10.7 µM NAA. Subculture of the tissues in medium enriched with 10% coconut water and 35.0 gL^{-1} ripe banana pulp resulted in the production of the highest average number of 40 shoots in 12 weeks. No difference in the regeneration potential was observed between the 3 young leaves, while mature leaves did not respond.

Ramos and Zamora (1990) studied on elimination of banana bunchy top infection from banana (*Musa* sp. cv. Lacatan) by heat pretreatment and meristem culture. They found that heat pre-treatment (40^0C for 16h photoperiod and 32^0C for 8 hours darkness) daily for 1 and 2 months of *in vitro* shoot cultures of BBTV infected banana cv. Lacatan before culture of isolated meristems yielded 59.3% and 62.5% BBTV symptom-free plants, respectively. Plants derived from shoot cultures established from infected corms, from shoot cultures which were heat treated for 1, 2, 4 and 8 weeks as well as from meristems derived shoots heat treated for 1 and 2 weeks all expressed symptoms of BBTV. Diseased plants were pale yellow and stunted during the shoot multiplication stage *in vitro*, typical symptoms appeared on diseased plants when potting out.

Paek *et al.* (1990) carried out experiment on the growth of rhizomes in a symbiotic seed culture following self or cross-pollination of cymbidium grown on MS medium. They reported that 3% banana homogenate to the medium generally favored rhizome growth. While 10% coconut milk favored shoot formation and growth. The results indicated that exposure to 10 mgL^{-1} BA for 10 20 days, followed by 0.5 mgL^{-1}

BA gave better shoot production than transfer to a medium with no BA or exposure to 10.0 mgL^{-1} for 30 or 40 days.

Besides shoot tip culture, callus induction, proliferation and slow growing cell suspension form mature fruits, leaf sheath primordia and rhizome tissue were also reported as ex-plant for banana (Ram and Steward, 1964). Although several investigators have attempted to establish callus and subsequently cell suspensions of bananas, they were equally unsuccessful in stimulating any organogenesis (Tongdee and Long, 1973; De-Guzman, 1975). Formation of spherical callus masses in *Musa* spp. resembling somatic embryo reported but no shoot regeneration was observed (Srinivasa *et al.* 1982; Bakry and Rossignol, 1985). Somatic embryo and subsequent recovery of plantlets were achieved by Escalant and Teisson (1989) culturing callus from zygotic embryos of a seeded diploid wild species and a hybrid.

Mateille and Foncelle (1988) described an improved micropropagation method for the Poyo banana clone in which 4 concentrations of Benzyladenine (BA) were used. They found that main apices of sucker bud and lateral bud were stimulated by high concentration of BA in absence of auxin. Proliferation was best achieved with 22.5 µM concentration of BA, reaching a multiplication rate of 2.8% every 3 weeks. After transplantation, shoot proliferation was achieved with the same concentration of cytokinin.

Hwang and Ku (1987) reported that among large numbers of Cavendish banana plants derived from meristem culture, about 3% were variants, where variant traits included stature, leaf shape, pseudostem colour and bunch characters. Meristem derived plants were screened for resistance to *F. oxysporium* f. sp. *cubense* by planting in (0-5% infection) were derived from 17979 plants screened. These were

inferior to the source cultivar for agronomic and fruit traits, but variation for these characters was found within each clone.

Bondok *et al.* (1987) conducted an experiment on production of virus free "Hindi" banana plants utilizing meristem culture and thiouracil treatments. They used shoot tips (0.5-1 mm) of banana plants naturally infected by Cucumber Mosaic Cucumovirus to obtain virus-free plants. Explants were cultured on Murashige and Skoog mineral salt medium with or without the antiviral compound (thiouracil). Plantlets were assayed for virus by serological diagnosis. The result showed that the regenerated plants were all virus free.

The fruit report on *in vitro* multiplication of edible banana and plantains was on the AAA genotypes in the early 1970 (Ma and Shii, 1972; Berg and Bustamante, 1974). Since then the number of reports have increased to include the AA and AAB genotypes (Cronauer and Krikonian, 1984a; Vuylseke and Langhe, 1985; Novak *et al.*, 1990) and the BBB group (Damasco and Barba, 1984; Jarret *et al.*, 1985). Banerjee *et al.* (1986) reported that shoot formation and rate of proliferation appear to be genotype dependent. They also found considerable difference between the rates of shoot proliferation in different cultivars.

Cronauer and Krikorian (1984b) noticed that established cultures from excised shoot tips of Grand Naine multiply rapidly. They raised single shoots on semi-solid medium and shoot clusters in liquid medium. Individual shoot was induced to form multiple shoot clusters by splitting the shoot longitudinally through apex. Shoot multiplication was maximum at 5.0 mgL^{-1} BAP.

2.2 Shoot proliferation and differentiation of banana

Ranjan *et al.* (2001) reported that the highest percentage of explants (shoot tip) responded to shoot initiation on MS media supplemented with 8.0 mgL^{-1} BA, 3.0 mgL^{-1} IAA and 150 mgL^{-1} adenine sulphate. The maximum numbers of shoots were 34.0, 32.3 and 30.3 for Dwarf Cavendish; Alpan and Batisa respectively, after 12-14 weeks of culture. The number of shoots per explant decreased with application of 9.0 mgL^{-1} BA.

For *in vitro* propagation of banana cv. Dwarf Cavendish, Gnand Nain, Petit Nain, Poyo, Williams and Basrai, meristem culture techniques were used by Gubbuk and Pekmezci (2001). They reported that different thidiazuron (TDZ) concentrations in combination with IAA were used in the propagation stage where TDZ concentrations of more than 2.5 μML did not affect the number of shoots and shoot quality. The results also pointed out the combination of TDZ and IAA was successful in terms of shoot height or length of shoot, and all the tested banana cultivars could be propagated via meristem culture. TDZ could be used as an alternative to benzyladenine in the propagation stage.

Pereira *et al.* (2001) carried out an experiment on the effect of different types of banana (cv. Prata) planting material where vegetative and reproductive plant development was cultured in lavras, Brazil. The results pointed out the type of planting material had an effect on growth characteristics, except that tissue cultured plantlets produced suckers earlier and in greater numbers than plants propagated from suckers of rhizome pieces.

Shoot tips were dissected from healthy suckers of banana (*Musa* spp) varieties dwarf Cavendish, Amruthapani, Tella Chakkerakeli and Robusta by Vani and Reddy (1999), and cultured on MS medium supplemented with 6.0 mgL^{-1} BAP, 2.0 mgL^{-1} IAA and 200.0 mgL^{-1} adenine sulphate. They found that shoot initials developed after 3 weeks and were proliferated on induction medium with BAP concentration reduced to 4.0 mgL^{-1}.

Khayat *et al.* (1999) carried out an experiment on banana germplasm improvement at Rahan meristem. Some 300 plants of Grand Naine banana (*Musa* spp) were propagated by meristem culture from 6 mother clones and also were evaluated for yield components at the Western Galilee Banana Experimental Station over 6 years. The results pointed out clones with superior yields were identified for propagation.

Jasari *et al.* (1999) found that 6-7 micro shoots were produced from the basal lateral sides of the main shoot tip, when cultured on modified MS medium supplemented with 6.0 mgL^{-1} BAP. Induction of roots was achieved on micro shoots when cultured on MS medium supplemented with IAA.

Azad and Amin (1999) cultured banana cv. Sabri using shoot tips (1.0-1.5 cm) of the field grown young suckers on MS medium supplemented with 3.0-5.0 mgL^{-1} BA + 2.0-3.0 mgL^{-1} IAA + 15% coconut water. Within 15 days of culture, the explants produced Tuber Like Structure (TLS) on above medium. When these TLS were longitudinally sectioned and sub-cultured on MS medium containing 1.0-2.0 mgL^{-1} BA, 0.5 mgL^{-1} IAA and 15% coconut water, those produced shoots with 3 to 5 leaves.

Bekheet and Saker (1999) prepared an efficient medium for *in vitro* propagation of banana cv. William, Grand Naine and Maghraby. Shoot cultures were established on MS medium supplemented with 2.0 mgL^{-1} BA, the shoots were then transferred to medium containing 0, 2.0, 4.0 or 6.0 mgL^{-1} BA for shoot multiplication. The highest number of proliferated shoots was recorded with 6.0 mgL^{-1} BA.

Shoot tips from suckers of the dwarf cultivars Basrai and Shrimanti were cultured on MS media supplemented with 5 mgL^{-1} BA for shoot multiplication by Nandi and Chaudhury (1997). Rooted plantlets were transferred to greenhouse for hardening at 22-24^{0}C and 90-95% RH. They concluded that there were no differences between the 2 cultivars. Nor were there any differences in growth of tissue cultured plants (number of roots, root length, height, or number of leaves) during the 2 month hardening period.

Following the first report of the *in vitro* production of meristem derived banana plants (Ma and Shii, 1972, 1974) shoot tip and meristem culture is now widely used in banana production. Abdullah *et al.* (1997) noticed that meristem tips with 2 pairs of leaf primordia from dessert banana clones GNGOA, SH 3362, William Highgete and Basrai rapidly proliferated shoots on MS medium containing 20.0 μML^{-1} BA. They also observed good tillering in all the genotypes.

A very high efficiency of *in vitro* shoot multiplication was recorded by Sharma *et al.* (1997) on MS semi-solid medium supplemented with 4.0 mgL^{1} IAA, 10.0 mgL^{-1} IBA and 200.0 mgL^{-1} Adenine hemisulphate. A maximum of 46.4 mean shoots were also obtained from their culture.

Rabbani *et al.* (1996) reported the effect of BAP and IBA on micropropagation of different banana cultivars viz. Amritasagar, Sabri, Anajee and Mehersagar, and observed that BAP at the rate of 5.0 mg/l produced the highest number of shoots in Amritasagar and Mehersagar. In another study, Amritasagar banana (AAA) meristem tip generated highest number of shoots on MS medium supplemented with 30 μM BAP (Khanam *et al.*, 1996).

Ali (1996) studied the effect of BAP and IBA on micropropagation of different banana cultivars viz Amritasagar, Sabri, Anajee and Mehersagar and observed that BAP at 5.0 mgL^{-1} produced the highest number of shoots in Amritasagar and Mehersagar.

Multiple shoots were induced from shoot tip culture on MS medium supplemented with BA (2.0 mgL^{-1}) by Ganapathy *et al.* (1995). Shoot tips excised from multiple shoots were sub-cultured on variety of media. It was also found that all components of MS medium were required for initial shoot multiplication.

Domingues *et al.* (1995) observed that explant of 1 cm long and 0.7 cm diameter obtained from banana cv. Maca gave the highest number of buds on nutrient solution containing 5.0 mgL^{-1} BA for 45 days.

Habib (1994) carried out an experiment on shoot multiplication of banana, and found that 5.0 mgL^{-1} BAP produced maximum plantlets from shoot tip explants.

Shoot multiplication rate for micropropagation of banana varies with the concentration of BAP present in MS basal media (Sun, 1985; Jarret *et al.*, 1985; Silayoi *et al.*, 1986; Namaganda, 1994). Kunlayanee *et al.* (1990) observed that shoot multiplication in 10.0 mgL^{-1} BAP produced more plantlets during culture of banana shoot tips.

Apical meristem were cultured by De-Gomez and De-Garcia (1994) on medium supplemented with 0.5 mgL^{-1} cysteine, 30.0 gL^{-1} sucrose, 8.0 agar and 0.5 or 1.0 mgL^{-1} BA during the initiation stage and with 5.0 or 10.0 mgL^{-1} BA and/or 0.25 or 0.5 mgL^{-1} BA at the multiplication stage. They found no differences among the cultures during the initiation stage with or without BA in the medium. But shoot proliferation was highest in the medium supplemented with 5.0 mgL^{-1} BA.

Tulmann Neto *et al.* (1990) found new buds of banana after shoot tip cultured *in vitro* on medium containing BA (5.0 mgL^{-1}), sucrose (30.0 gL^{-1}) and agar at pH 5.7. The multiplication rate of banana cv. Poyo micro propagated *in vitro* in a liquid medium was 4-5/explant per 20 day cycle (Cote *et al.* 1990) compared to a multiplication rate of 1.5-3.0 for the Cavendish group when grown commercially on a solid medium. However, altering apical dominance of the explant by cutting the meristem significantly increased the range.

Raut and Lokhande (1989) studied the effect of different concentration of BAP on multiple shoot and single shoot formation. When *Musa paradisica* cv. Basrai cultured on MS medium supplemented with 7.0 or 10.0 mgL^{-1} BAP multiple shoots were formed. On the other hand, they obtained single shoots when the explants were cultured on MS medium containing 5.0 mgL^{-1} BAP.

Cronauer and Krikorian (1985, 1986), Doreswamy and Shahijram (1989) observed that aseptic shoot cultures can be established from floral apices. In floral apices, shoot formation began with the appearance of scars or bud like structures in the axil of bract that subsequently developed into shoots, morphologically identical to those originating from vegetative apices (Cronauer and Krikorian, 1988). Bakry *et al.*

(1985) found that the morphology of the newly developed shoots was dependent on the stage of floral differentiation.

Balakrishnamurthy and Rangasamy (1988) conducted an experiment and found that terminal male flower buds of the varieties Robusta and Montana when cultured on MS medium supplemented with 30.0 gL^{-1} sucrose, 0.8% bactoagar and 0, 2.5, or 5.0 mgL^{-1} BAP, all the floral apices survived. But proliferation of buds was observed only in culture medium containing BAP at 2.5 and 5.0 mgL^{-1} for both the varieties.

Fitchet and Winnar (1988) reported that *in vitro* shoot tip culture of the cultivars dwarf Cavendish and Williams gave the best development when cultured on media supplemented with a mixture of IBA (2.0 mgL^{-1}), NAA (2.0 mgL^{-1}), kinetin (5.0 mgL^{-1}) adenine sulphate (160.0 mgL^{-1}), Sodium phosphate (340.0 mgL^{-1}) and activated charcoal (5.0 gL^{-1}). They also found that 10% calcium hypochlorite gave the best result as surface sterilant for decontamination of shoot tips among several surface sterilants used.

In case of micropropagation of banana, different types of explants such as offshoots, rhizomes or aerial organs (leaf, pseudostem or fruit) may be used. Kamate and Anno (1987) investigated meristems from some tissues. They found that only the meristem developed into plants without callus formation and up to 5 plants could be obtained from a meristem.

Banerjee *et al.* (1987) cultured thin meristematic layers excised from proliferating shoot tips of *Musa* sp. cv. Bulggoe (ABB) and found callus when cultured on MS basal medium supplemented with 2,4-D or 2,4,5-T. On prolonged

incubation, the callus developed numerous globular white proembryogenic masses all over the surface. Upon transferring to MS liquid medium devoid of 2,4-D and 2,4,5-T but supplemented with other auxin and cytokinin, the proembryoids turned greenish. The proembryoids obtained from 2,4-D containing medium produced only roots while those from a 2,4,5-T containing medium regenerated bipolar embryos.

In separate experiments, Ma and Shii (1972), and Doreswamy *et al.* (1983) found that decapitation of the apical dome was necessary for the release of new shoots. Destruction of the central growing point allows axillary buds to develop. These were normally suppressed through apical dominance (Cronauer and Krikorian, 1986). Wong (1986) discussed the conflicting results of Ma and Shii (1972) with those of Doreswamy *et al.* (1983) and supported the idea that multiple shoot formation could also be achieved when the apical dome was not destroyed and a cytokinin source was included in the medium.

Gupta (1986) reported that heat therapy and meristem culture were good for rapid clonal propagation of mosaic disease free banana plants. Suckers were subjected to heat therapy at 38-40^{0}C for 14 days prior the culture of their meristem tips on modified MS medium containing 1.0 mgL^{-1} thiamine HCl, 0.5 mgL^{-1} nicotinic acid, 0.5 mgL^{-1} Pyridoxine HCl, 25 mgL^{-1} ascorbic acid, 0.7 mgL^{-1} BA and 0.7 mgL^{-1} Kinetin within 10 to 12 weeks. A single heat treated shoot tip produced up to 13 rooted plantlets and were free from diseases.

Vuylsteke and Langhe (1985) reported rapid clonal propagation of banana and plantains *in vitro* from meristem tip. Damasco and Barba (1984) observed that corm sections and tissue culture derived shoots of Saba banana (*Musa* sp. cv. Saba BBB) formed multiple shoots on MS medium supplemented with 10.0 mgL^{-1} BA. They also

observed that subculture of shoot tip to fresh medium of the same composition increased the number of shoots produced in each culture cycle.

Shoot multiplication rate during micropropagation of banana varies with the concentration of BAP present in MS basal media (Sun, 1985; Jarret *et al.* 1985). Proliferation rate was maximum when the culture medium was supplemented with different vitamins, organic compounds and 5.0 mgL^{-1} BAP.

2.3 Establishment of *in vitro* banana plants in rooting media

In tissue culture, rooting of banana plantlets is very important. A separate root induction phase is essential for rooting of banana shoots before transferring them into soil. Various auxins at different concentrations are capable of root induction in micropropagated banana plantlets.

Habiba *et al.* (2002) observed that half strength MS supplemented with 2.0 mgL^{-1} IBA was the best for root induction in the regenerated shoots.

An *in vitro* production of tetraploid banana plantlets (*Musa sp.* FHIA01 AAA group) was developed and the cloning effect on the *in vitro* development of the explants, the rate of contamination, multiplication and BAP concentrations were studied by Oliveira *et al.* (2001). They showed that higher multiplication rates were obtained, averaging 2.65 per sub-culture, on the MS media supplemented with 4.0 mgL^{-1} BAP. A pronounced effect of the cloning on multiplication was observed. Somaclonal variations were not observed among *in vitro* plantlets.

Gubbuk and Pekmezci (2001) reported that the use of 1.0 μML^{-1} IBA or NAA with MS medium was sufficient in terms of rooting.

Vani and Reddy (1999) reported that the shoots were rooted on MS medium containing 2.0 mgL^{-1} BAP, 2.0 mgL^{-1} IAA and 0.1% activated charcoal and eventually established in soil.

Azad and Amin (1999) observed that isolated plantlets of banana var. Sabri produced from shoot tips, when cultured on MS medium supplemented with 1.0-2.0 mgL^{-1} IBA + 0.5-1.0 mgL^{-1} IAA + 15% coconut water induced rooting *in vitro* on MS medium with 0.2-0.5 mgL^{-1} IBA.

Abdullah *et al.* (1997) noticed that shoots of GN60, SH 3362 William high gate and Basrai easily rooted on MS medium containing 4% sucrose and 1.0 μML^{-1} IBA.

Nandi and Chaudhury (1997) found that the MS medium supplemented with 5.0 mgL^{-1} IBA was the best for root differentiation of the dwarf cultivars Basrai and Shrimanti.

Khanam *et al.* (1996) reported shoot developed from meristem tip culture of Amritasagar banana on MS medium supplemented with 30.0 μML^{-1} BAP. Rabbani *et al.* (1996) observed that shoots derived from MS culture medium produced maximum number of roots in cultivar Amritasagar on MS medium supplemented with 2.0 mgL^{-1} IBA. In liquid, Domingues *et al.* (1995) observed that rooting was stimulated in a nutrient solution supplemented with 0.1 mgL^{-1} NAA or IBA.

Raut and Lokhande (1989) found that rooting was induced on MS medium supplemented with 2.0 mgL^{-1} IBA and whole plantlets were successfully established

in pot. Micropropagated banana plantlets produced sufficient roots in the medium containing half strength MS media supplemented with 2.0 mgL^{-1} IBA (Habib, 1994).

Bhaskar *et al.* (1993) found that *in vitro* rooting for 2 to 5 cm long shoot explant were best (6.75 roots per shoots within 6 days) on Knudsen's medium supplemented with 5.0 ppm NAA.

Devi and Nayar (1993) reported that roots were induced within 4-5 days of culturing single shoots on MS medium containing 0.25% charcoal and 0.1 μML^{-1} IBA. The plantlets were potted in vermiculite 2-3 weeks after rooting and were successfully transplanted to the field within 3 months.

Seeni and Latha (1992) observed that meristem derived shoots readily formed roots when transferred to a medium containing 4.4 μML^{-1} BA, 10.7 μML^{-1} NAA and 1% activate charcoal.

Murali and Duncan (1991) observed that basal medium supplemented with 1.0 mgL^{-1} IBA induced root in micropropagated shoots of banana. Cronauer and Krikorian (1984b) obtained rooted plantlets by treating with NAA (1.0 mgL^{-1}) and activated charcoal (0.02%).

Lameira *et al.* (1990) observed that shoots obtained from explants of variety Prata (AAB) cultured on MS medium with BA, initiated roots on half strength MS medium with 2.0 mgL^{-1} IBA. They also observed that plantlets grow on medium containing vermiculite, organic matter and plant nutrient without growth regulators.

Mateille and Foncelle (1988) reported that shoot elongation and rooting of banana cv. Poyo occurred simultaneously on a medium containing 10.0 g/L sucrose

and no phytohormone. They also found that rooting was improved when the bottoms of the culture tubes with medium were set in darkness.

Fitchet and Winnaar (1987) observed that 4 weeks old shoot induced more roots in semisolid rooting medium of IBA, NAA, kinetin and activated charcoal. They also observed that rooted plantlets were successfully established on soil in 3 weeks.

Gupta (1986) reported that regeneration of shoot clusters and subsequently rooted plantlets from meristem tip took 10 to 12 weeks on BAP and kinetin enriched MS medium.

Molla *et al.* (2004) reported shoot tip of BARI banana 1 were culture on MS medium supplemented with 5.0 mgL^{-1} BAP for shoot proliferation. Well developed shoots were used for rooting. Among the six different concentrations of IBA (0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 mgL^{-1}) in half strength MS media, a good number of healthy roots were produced on 0.5 mgL^{-1} IBA (6.89 and 0.4 mgL^{-1} IBA (6.31).

2.4 *Ex vitro* survival of plantlets

The survival rates of *in vitro* grown plantlets depend on the successful establishment of those under *ex vitro* conditions –

Karmarkar *et al.* (2001) carried out an experiment on *in vivo* cultures and *in vitro* plant materials (suckers and harden plants) of banana cv. Basrai (AAA). These plants were exposed to 60 co gamma ray doses ranging from 0-100 GY. Radio sensitivity of suckers and *in vitro* individual shoots was assessed by recording data on survival, multiplication ratio, days to root initiation, root number, shoot length, root length, plant height, number of leaves, leaf area and chlorophyll content. The results

obtained from the *in vivo* and *in vitro* plant materials exhibited differential response to gamma irradiation.

A protocol was described by Dilip *et al.* (2000) for large-scale multiplication and *ex vitro* survival of banana var. 'Basrai' through tissue culture. Multiple shoot cultures from shoot tip ex plants of 'Basrai' were established on modified Murashige and Skoog (MS) medium. They found that maximum 28 shoots could be achieved on MS medium containing KH_2PO_4 (170.0 mgL^{-1}), Ascorbic acid (50.0 mgL^{-1}), Adenine hemisulphate (100.0 mgL^{-1}), Benzyladenine (100.0 mgL^{-1}), Kinetin (3.0 mgL^{-1}) and Indole acetic acid (0.5 mgL^{-1}). They also reported that extending subculture cycle 8 or 9 showed variants in culture. Maximum (90%) rooting was achieved in well developed shoots on MS medium supplemented with Indolebutyric acid (1.0 mgL^{-1}). More than 25,000 plantlets produced through tissue culture were transferred to field with 90% survival rate.

The well developed plantlets were hardened without greenhouse facilities for their establishment in the field by Jasari *et al.* (1999). The results showed that average 92% of the plantlets survived hardening with the protocol developed.

Azad and Amin (1999) stated that rooted plantlets of banana cv. Sabri produced *in vitro* were successfully established on pot mixture containing sand, soil and compost at 1:1:1 ratio. They also mentioned that survival of the plantlets under *ex vitro* condition was 80%.

An investigation on evaluation of commercial micropropagation of banana was carried out by Oliveira *et al.* (1997). They observed that losses of *in vitro* grown plantlets due to contamination were 18% and 40.60% in two cultivars Nanicao and

Grand Naine, respectively whereas losses during acclimatization were 2% in 80 days (60 days covered with polythene bags and 20 days outdoors) when rooted plantlets were transferred to the medium containing organic matter: sand: soil (1:1:1).

Nandi and Chaudhury (1997) reported that there were no differences between the two cultivars in terms of growth of tissue-cultured plants (number of roots, root length, height or number of leaves) during the two-month hardening period.

Daniells *et al.* (1995) screened banana cv. Mysore was for female fertility. A triploid hybrid was found to contain BSV, which confirmed seed transmission of the virus. Attempts were under taken to free Mysore from the virus by meristem culture.

De-Gomez and De-Garicia (1994) observed that ten months after transferring to the soil, plantlets obtained form media supplemented with 10.0 mgL^{-1} BA were larger and had longer petioles than plantlets obtained form media supplemented with 5.0 MgL^{-1} BA during the multiplication stage.

A comparative study was conducted by Kawit *et al.* (1993) on the performance of tissue culture propagated bananas and conventional sucker planting bananas of 16 cultivars in Thailand. The results pointed out that tissue culture plants could be used in commercial banana production in Thailand with some advantages.

An investigation on field evaluation of tissue cultured bananas in South Eastern Queens land was carried out by Drew *et al.* (1990). They observed that tissue cultured plants established more quickly were taller, and had a shorter time to bunch emergence than the conventional planting materials. They had significantly higher yields in term of bunch weight, which was a function of greater number of fingers and hands. The results also pointed out that 22% of the plants derived from callus culture

were off types, compared with 3% in the line produced by axillary bud production. No off type plants were observed in conventional planting material.

Gupta (1986) reported that the survival of plantlets on transfer from *in vitro* cultures to soil was more than 95%. He also noted that the meristem derived plants grew faster and facilitated early harvesting compared to conventionally propagated suckers. Moreover, plant height at maturity and fruit productivity were almost equal among the plants of both origins.

Cronauer and Krikorian (1984a) mentioned that *in vitro* induced rooted shoots of four banana cultivars were successfully established on pot mixture containing soil and vermiculite at 1:1 ratio and also stated that survival from culture vessel to soil was 100%.

Molla *et al.* (2004) reported that plantlets transferred to plastic pots after 10, 15, 20, 25 and 30 days *in vitro* culture were survived 95-100% plantlets whey they were transferred after 15-20 days *in vitro* culture with 7 days hardening at room temperature.

From the above review, it was evident that different plant growth regulators (PGRs) at different concentrations individually or in combinations influence *in vitro* culture of banana plants. However, very few investigations have yet been carried out on these factors.

CHAPTER III

MATERIALS AND METHODS

The present study was planned during September, 2004 to June, 2005 at the Biotechnology laboratory, Biotechnology Division, Bangladesh Agricultural Research Institute (BARI), Joydebpur, Gazipur. Materials and methods used to conduct the present study have been presented in this chapter.

3.1 Experimental materials

3.1.1 Plant materials

The planting materials of banana cv. BARI-1 were collected from Biotechnology Division, Bangladesh Agricultural Research Institute (BARI), Joydebpur, Gazipur. The meristem used for establishment of culture was prepared under the microscope from the collected suckers through dissection and removal of leaf sheath.

3.1.2 Culture media

The degree of success in any technology employing cell, tissue and organ culture is related to relativity few major factors. A significant factor is the choice of nutritional components and growth regulators. Murashige and Skoog (1962) medium supplemented with different phytohormones as per treatments were used as culture medium for shoot induction, shoot multiplication and maintenance and regeneration of roots from multiplied shoot. The composition of MS medium has been presented in Appendix-I. Hormones were added separately to different media according to the requirements. For the preparation of media, stock solutions were prepared at the

beginning and stored at 9±1^{0}C temperature. The respective media were prepared from the stock solutions.

3.2 Preparation of the stock solution

The first step in the preparation of the medium was the preparation of stock solutions. The various constitutes of the medium were prepared into stock solutions for ready use to expedite the preparation of the medium. Separate stock solutions for macronutrients, micronutrients, irons, vitamins, growth regulators etc were prepared and used.

3.2.1 Stock solution A (Macronutrients)

The was made up to 10 folds the final strength of the medium in 1000 ml of distilled water (dw). Ten times weight of salts required per litre of the medium were weighed accurately and dissolved in 750 ml of dw and final volume was made up to 1000 ml by further addition of distilled water. This stock solution was filled and poured in to a clean brown bottle and stored in a reregister at 9±1^{0}C for further use.

3.2.2 Stock solution B (Micronutrients)

Stock solution of micronutrients was made up to 100 folds the final strength of the medium in 1000 ml of dw as described earlier for stock solution A. The stock solution was filtered, labeled and stored in a refrigerator at 9±1^{0}C for further use.

3.2.3 Stock solution C (Iron source)

It was made up to 100 folds the final strength of the medium in 1000 ml of dw. Here two constitutes, $FeSO_4$ and Na-EDTA were dissolved in 750 ml of dw in a **beaker by heating on a heater cum magnetic stirrer. Then the volume was made up to 1000 ml further addition of dw. Finally, the stock solution was filtered and stored by wrapping with aluminium foils in a refrigerator at 9±1^{0}C for further use.**

3.2.4 Stock solution D (Vitamins and amino acids)

Each of the desired ingredients except myoinositol were taken at 100 folds (100x) of their final strength in a measuring cylinder and dissolved in 400 ml of dw. Then the final volume was made up to 1000 ml by further addition of dw. Finally the stock solution was filtered and stored in a refrigerator at 9±1°C for later use. But the myoinositol was made separately 100 folds (100x) the final strength of the medium in 1000 ml of dw. This stock solution was also filtered and stored in a refrigerator at 9±1°C.

3.2.5 Stock solution E (Growth regulators)

In addition to the nutrients, it is generally necessary to add growth regulators (hormones) such as auxin and cytokinin to the medium to support good growth regulators were used in the present investigation, which were dissolved in appropriate solvent as shown each of them.

Plant growth regulator (Solute)	**Solvent**
Auxins	
Indole - 3 acetic acid (IAA)	70% ethyl alcohol
Indole butyric acid (IBA)	70% ethyl alcohol
A – naphthalene acetic acid (NAA)	0.1 N NaOH
Cytokinin	
6 – Benzyl amino Purine (BAP)	0.1 N NaOH

To prepare a stock solution of any of these growth regulators, 10 mg of the growth regulator was taken on a clean watch glass then dissolved in 1 ml measuring cylinder and the volume was made up to 1000 ml with distilled water. The solution was then poured into a clean glass container and stored at $9\pm1^{0}C$ and used for a maximum period of two months.

3.3 Media Preparation

i. One hundred milliliter (ml) of macromutrients, 10 ml of micronutrients, 10 ml of irons, 10 ml of vitamins and 10 ml of myoinositol were taken from each of these stock solutions in to a 2-litre beaker on a hot plate magnetic stirrer.

ii. Five hundred ml distilled water was added in to the beaker to dissolve all the ingredients.

iii. Thirty gram of sucrose was added to this solution and gently agitated to dissolve completely.

iv. Different concentrations of hormonal supplements as required added either in single or in different combinations to this solution and were mixed thoroughly.

v. Since each hormonal stock contained 10 mg of the chemical, in 100 ml of solution, to make one litre of medium addition of 10 ml of stock solution of any of the hormones, resulted in 1.0 mgL^{-1} concentration of that hormonal supplement. Similarly for 0.5, 1.0, 1.5, 2.0, 2.5, 5.0, 7.5 and 10.0 mgL^{-1} concentration of the hormonal supplement 5, 10, 15, 20, 25, 50, 75 and 100 ml of hormonal stock solutions were added, respectively to prepare one litre of medium.

vi. pH of the medium was adjusted to 5.8 by pH meter with the help of 0.1 N NaOH or 0.1 N HCl whichever was necessary.

vii. After adjusting the pH, 7.0 gL^{-1} agar was added to solidify the medium. The mixture was then heated for 15 minutes in a microwave oven and stirred till complete dissolution of agar.

viii. Required volume of hot medium was dispensed into culture vessels or test tubes. After dispensing the medium the test tubes were covered with aluminium foil and marked with different codes by the help of a glass marked to indicate specific hormonal combination.

3.4 Sterilization of media

The culture tubes with media were then autoclaved at 1.06 kg/cm^2 pressure at 121^{0}C for 25 minutes. The medium was then cooled at room temperature before use.

3.5 Sterilization of glassware and instruments

Culture tubes, beakers, pipettes, measuring cylinder, metal instruments such as forceps, scalpel, needles, spatula and aluminium foils were sterilized in a pressure cooker or in a autoclave at a temperature of 121^{0}C for 20 minutes at 1.06 kg/cm^2 pressure.

3.6 Sterilizing culture room and transfer area

Initially, the culture room was cleaned by gently washing all floors and walls with detergent or Lysol (germicide). This was followed by carefully wiping them with 70% ethyl alcohol. The process of sterilization of culture room was repeated at regular intervals.

Laminar air flow cabinet was usually sterilized by wiping the working surface with 70% ethyl alcohol and switching on the uv light of the cabinet for 30 minutes before starting the transfer work. After the switching off on the uv light 15 minutes duration kept for safety to stark work.

3.7 Precaution to ensure aseptic conditions

The cabinet was usually started half an hour before use and wiped with 70% ethyl alcohol to reduce the chances of contamination. The instruments like forceps, scalpel, needles etc were pre-sterilized by autoclaving and subsequent sterilization was done by dipping in 70% ethyl alcohol followed by flaming and cooling method inside the laminar air flow cabinet while not in use, the instruments were kept inside

the laminar air flow cabinet. Hands were also sterilized by wiping with 70% ethyl alcohol. Aseptic conditions were followed during each and every operation to avoid the contamination of culture.

3.8 Culture method

The following culture methods were employed in the present investigation:

i. Explant culture and

ii. Subculture or transfer.

3.8.1 Explant culture

3.8.1.1 Preparation of explants

The meristem was the starting material. It was obtained from developing suckers (about four months of age) of banana cv. BARI-1 grown under field conditions and was brought to the preparation room (Plate 1). The suckers were washed thoroughly under running tap water. The roots and outer tissues of the suckers were removed with the help of a sharp knife (Plate 2). A number of outer leaves were removed until the shoot measured about 1.0 – 2.0 cm in length and 1.0 cm width at the base. Then the initial explant was prepared under stereomicroscope by removal of outer tissue of meristem with the help of sterile scalpel, which was about 5×5 mm in size.

Plate 1. Explants collected from field 3-4 months aged banana plant

Plate 2. Banana explants prepared for the meristem collection.

3.8.1.2 Surface sterilization of explants

The pale white tissue block (1.0×2.0 cm) containing meristem and rhizomatous base were taken in a beaker. Surface sterilization is done under laminar Airflow Cabinet with 70% ethyl alcohol, the explants were surface sterilized with 0.1% mercuric chloride and a few drops of Tween 20 for 15 minutes. Finally, the explants were then rinsed three to four times with sterile dw.

3.8.1.3 Inoculation of culture

The isolated and surface sterilized explants meristems were collected carefully under the stereomicroscope through maintaining aseptic condition inside the laminar air flow cabinet, to use those as explants. The individual meristems were directly inoculated to each of the culture tube containing 20 ml of MS medium supplemented with different concentration of hormones as per treatment covered with aluminium foil.

3.8.1.4 Incubation

The culture tubes were transferred to growth room and allowed to grow in controlled environment. The temperature of the growth room was maintained with in $25\pm1^{0}C$ by an air conditioner. A 16 hour light period was maintained with light intensity of 2000 lux of the growth and development of culture.

3.8.1.5 Blackening of the explant

Some explants become black in color within 6-7 days after inoculation. To control blackening after about one week the blackish tissues on the explants were removed and the meristematic tissues were transferred to similar fresh medium. It was repeated each of 10 days interval for about one month to minimize further blackening of the tissues.

3.8.2 Sub culture or transfer

3.8.2.1 Maintenance of proliferating shoots

Initial subculturing was done when the explant had produced some shoots. For sub-culturing, the entire samples of *in vitro* shoot were cut into small pieces so that each piece would contain about one shoot. Leaf and blackish or brownish basal tissues were removed to expose the meristems. Each piece was inoculated into a similar fresh medium. It was practiced at the interval of every one month.

3.8.2.2 Regeneration of plants from *in vitro* proliferated buds

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

3.8.2.3 Root induction of regenerated shoots

When the shoots grew about 3-5 cm in length with 3-6 well developed leaves they were rescued aseptically from the culture tubes and were separated from each other and again cultured on freshly prepared medium containing different combinations of hormonal supplements for root induction.

3.9 Preparation of pot

Potting mixture containing ground soil and cowdung in the ratio of 1:1 was mixed thoroughly and were placed into a 10×15 cm polythene bag for growing *in vitro* grown plantlets under *ex vitro* conditions.

3.10 Treatments

Two experiments were conducted to asses the effect of different concentrations of BAP, NAA, IAA and IBA on shoot proliferation and subsequent rooting of the multiplied shoot.

Experiment 1. Effect of different concentration and combination of BAP and NAA on multiple shoot proliferation from meristem cultured explant

In this experiment, *in vitro* derived banana cv. BARI-1 plantlets were used as sources of meristem to investigate the effect of BAP, NAA each at different concentration alone or in combinations on shoot proliferation.

Treatments: 5 levels of BAP (0.0, 2.5, 5.0, 7.5, and 10.0 mgL^{-1}) and 5 levels of NAA (0.0, 0.5, 1.0, 1.5 and 2.0 mgL^{-1}) were used. All the combinations of both BAP and NAA were used as treatments.

Experiment 2. Effect of different concentration of IAA and IBA on root formation of the micro-propagated shoots was investigated.

There were 3 levels of IAA (0.0, 0.5 and 1.0 mgL^{-1}) and 4 levels of IBA (0.0, 0.5, 1.0, and 1.5 mgL^{-1}) were used as treatments. The experiments were arranged in Completely Randomized Design (CRD) with 4 replications. Each of replications consisted of 10 culture tubes.

3.11 Data collection

Data were collected on the effect of different treatments on shoot proliferation and rooting. The following parameters were recorded.

3.11.1 Percentage of explants induced shoot

Excised explants were used for shoot initiation. Regeneration of explant was recorded at 10 day interval up to one month of culture. Percentage of explant induced shoots was calculated using the following formula.

$$\% \text{ explants induced shoot} = \frac{\text{Number of explant induced shoot}}{\text{Total number of explants inoculated}} \times 100$$

3.11.2 Number of shoots per explant

Number of shoots per explant was recorded at 10 day interval up to one month of culture and mean number of shoots per explant was calculated by using the following formula, $X = \frac{\sum x_i}{n}$

Where,

X = Mean number of shoots/explant

X_i = Number of shoots/explant

n = Number of observation

3.11.3 Length of longest shoot

Length of longest shoot was measured in centimeter (cm) from the base to the top of the explants by a measuring scale. It was calculated at 10 days interval up to one month of culture and the mean was calculated.

3.11.4 The average number of leaves and length of longest leaves

Number of leaves produced on the plantlet and length of the longest leaf were counted at 10 days interval up to one month of culture and the mean was calculated.

3.11.5 Percentage of shoot developed roots

Excised shoots were used for root induction. The percentage of shoots induced roots were calculated using the following formula.

$$\% \text{ shoot induced root} = \frac{\text{Number of shoot induced root}}{\text{Total number of shoots inoculated}} \times 100$$

3.11.6 Number of roots per plantlet

Average number of roots per plantlet was counted at 10 day's interval up to one month of the culture and the mean was calculated.

3.11.7 Length of roots

Root length was measured in centimeter from the base to the tip of the roots at 10 days interval up to one month of the culture. Average length of the root was calculated using the formula as mentioned earlier.

CHAPTER IV

RESULTS AND DISCUSSION

Regeneration of banana plantlets through meristem culture offers the unique facilities of reproduction protocol with a view of disease free planting material such as bunchy top, cucumber mosaic virus and panama wilt. So, the present series of work were conducted at Biotechnology Division of BARI with the variety of BARI-1 of banana using meristem as explant and subsequent shoot regeneration and micropropagation.

In this experiment BAP (0.0, 2.5, 5.0, 7.5 and 10.0 mgL^{-1}) and NAA (0.0, 0.5, 1.0, 1.5 and 2.0 mgL^{-1}) were used as treatments. The results of the experiment are presented in Table 1-6 and discussed in this chapter. Analysis of variance in respect of all the parameters have been presented in Appendix 2-19.

4.1 Regeneration of shoot from meristem explants

The meristem was excised under a sterio binocular microscope and transferred to solid meida. *In vitro* culture of meristem (Plate 3) results hard meristematic ball like structure in regeneration media containing different concentration of BAP and NAA (Plate 4). The culture meristem first turned brown in colour in 4-5 days and after 30-50 days later a green globular hard coat mass grow to be round in shape producing a ball like structure. From this ball like structure adventitious plantlets were developing (Plate 5).

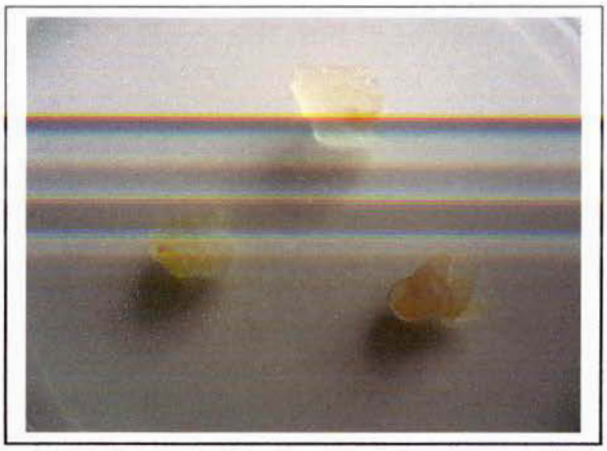

Plate 3. The excised meristem of banana cv. BARI-1 ready for inoculation on culture media

Plate 4. Green globular hard coat meristematic ball like structure produced from cultured meristem in the media containing different concentration of BAP and NAA at 30 days after inoculation

Plate 5. Adventatious shoot regeneration initiation from meristematic hard coat structure on MS medium supplemented with different concentration of BAP and NAA at 30 days after inoculation

Rahaman *et al.* (2004) observed that hard ball like structure developed from meristem explant in MS media supplemented with 5.0 mgL^{-1} BAP. They also notice that single shoot regeneration from meristem explant was thinner than shoot derived from shoot tip. Rabbani *et al.* (1996) denoted that 2 ip have the more in producing ball like structure. Similar results also obtained by Habib (1994) and Ali (1996) in their experiments. They observed that some ball like structures formed from the base of the shoot during shoot multiplication. These ball like structures are suitable for *in vitro* germplasm conservation.

4.1.1 Experiment 2. Effect of different concentrations and combination of BAP and NAA on multiple shoot proliferation from meristem derived explant.

Plant regeneration and subsequent shoot multiplication from meristem derived plantlets of banana cv. BARI-1, MS medium supplemented with different concentrations of BAP and NAA was used and healthy and vigorous explants were collected from the meristem derived plantlets and cut into pieces half rhizome, a piece of 2.5-3.5 cm long and about 2 cm diameter were used as primary culture (explant) for shoot multiplication and proliferation.

The results obtained from this experiment have been presented in Table 1-4 and discussed under the following headings.

4.1.1.1 Number of shoots per explant

The number of shoots produced per explant was varied in MS media supplemented with different concentrations of BAP and NAA. Data were recorded at 10, 20 and 30 Days After Inoculation (DAI) and results have been presented in the Table 1.

Table 1. Effect of different concentrations of BAP and NAA on shoot multiplication of banana plantlet cv. BARI-1 at different days after inoculation

Treatments		Number of shoots		
BAP (mgL^{-1})	NAA (mgL^{-1})	10 DAI	20 DAI	30 DAI
0	0	0.0 b	1.00 c	1.00 f
	0.5	1.0 a	1.50 bc	2.25 def
	1.0	0.5 ab	1.25 bc	2.50 de
	1.5	1.0 a	1.50 bc	1.50 ef
	2.0	0.75 a	1.25 bc	2.00 def
2.5	0	1.0 a	1.00 c	1.50 ef
	0.5	1.0 a	1.25 bc	1.75 ef
	1.0	1.0 a	1.25 bc	2.50 de
	1.5	1.0 a	1.75 abc	1.75 ef
	2.0	1.0 a	1.50 bc	2.25 def
5.0	0	1.0 a	1.25 bc	1.50 ef
	0.5	1.0 a	1.75 abc	2.50 de
	1.0	1.0 a	2.25 ab	2.50 de
	1.5	1.0 a	1.50 bc	2.50 de
	2.0	0.75 a	1.50 bc	1.75 ef
7.5	0	0.75 a	1.75 abc	2.50 de
	0.5	0.75 a	2.75 a	6.25 a
	1.0	0.75 a	2.75 a	5.25 ab
	1.5	1.0 a	1.75 abc	4.25 bc
	2.0	1.0 a	1.75 abc	2.25 def
10.0	0	1.0 a	1.25 bc	2.25 def
	0.5	0.75 a	2.00 abc	2.50 de
	1.0	0.75 a	2.00 abc	2.50 de
	1.5	0.5 ab	1.50 bc	3.25 cd
	2.0	0.75 a	1.50 bc	2.50 de
LSD value (0.01)		0.61	1.05	1.26
CV (%)		38.88	34.55	26.71

The effect of different concentrations of BAP and NAA on shoot regeneration and proliferation were statistically significant at 1% level of significance. Among the different concentration 7.5 mgL^{-1} BAP + 0.5 mgL^{-1} NAA showed highest shoot proliferation 0.75, 2.75 and 6.25 shoots per explant at 10, 20 and 30 DAI respectively. Followed by 7.5 mgL^{-1} BAP + 1.0 mgL^{-1} NAA treatment (0.75, 2.75 and 5.25 shoots per explant at 10, 20 and 30 DAI respectively. A good number of shoot proliferation was achieved at 7.5 mgL^{-1} BAP + 1.5 mgL^{-1} NAA at 30 DAI (4.25) which is superior from the control treatments (1.00). The regeneration and proliferation of shoots was sequentially described in plate 6, 7, 8 and 9 from initial sub culture to 4th sub culture.

Rahaman *et al.* (2004) found height shoot number at 1.5 mgL^{-1} BAP + NAA (4.52 explant) at 30 DAI. The result of current investigation is not fully supported by Rabbani *et al.* (1996) where they found that the highest number of shoots per explants at 28 DAI (3.11 ± 0.66) with 5.0 mgL^{-1} of BAP and Kn. This variation might be due to the different concentration of NAA (auxins) and BAP (cytokinin) and their associations.

Olivia and Barba (1984) obtained 10.10 number of shoots in the optimum concentration of 10.0 mgL^{-1} BA. They also found that increasing results in the proliferation of shoots in the increase of cycles of culture (first cycle 11.32 and 4th cycle 17.78 number of shoots). Doreswamy *et al.* (1983) stated 20-25 shoot bud and 35 shoot lets in Ms + 10 mgL^{-1} + 15% CM.

The explants cultured on MS medium without growth regulator produces only single shoots at 20 and 30 DAI. This findings does not encourage with the results of Khanam *et al.* (1996), Rabbani *et al.* (1996) and Rehana (1999) where they did not monitor any shoot formation in case of control treatment. This study complies that if the explant in the culture media does not contaminated by fungus or bacteria than the explant develop only single shoot in long culture. The large variation in number of shoots per explants observation may be due to the genotype, chemicals and culture environments.

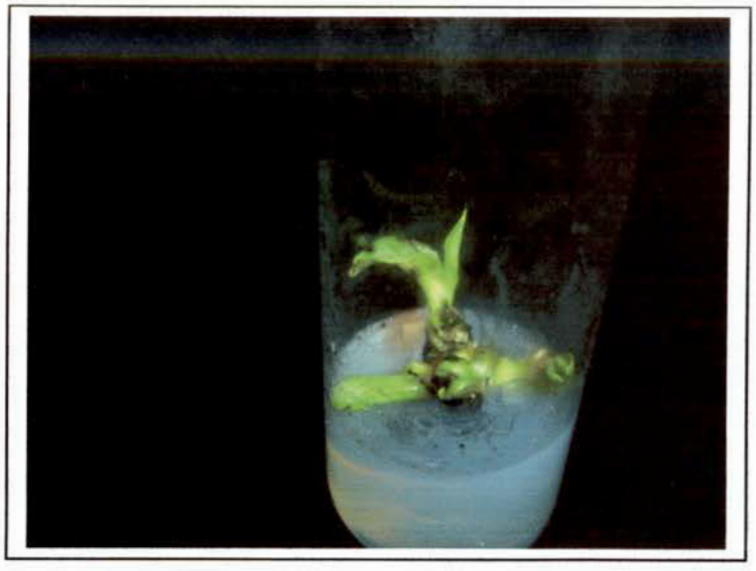

Plate 6. Multiple shoots produced from meristem explant cultured on MS medium supplemented with 7.5 mgL^{-1} BAP + 0.5 mgL^{-1} NAA at 30 days after inoculation

Plate 7. Multiple shoots produced from meristem explant cultured on MS medium supplemented with 7.5 mgL^{-1} BAP + 0.5 mgL^{-1} NAA at 2^{nd} subculture

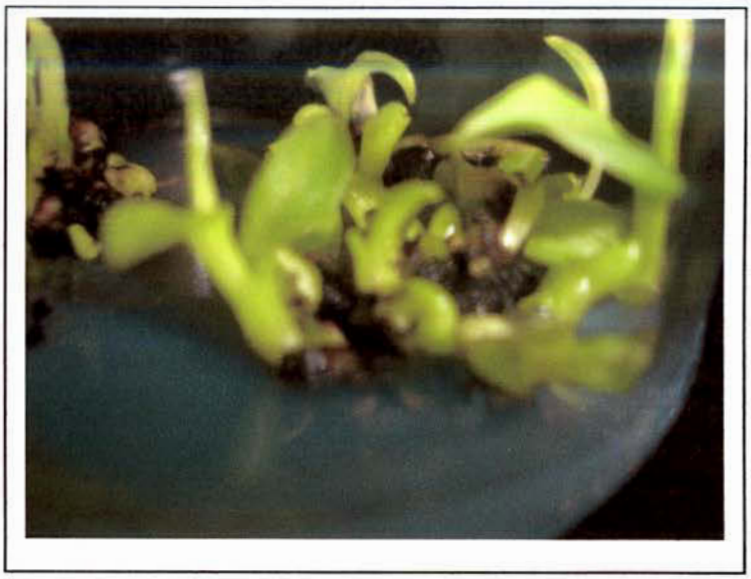

Plate 8. Multiple shoots of banana produced on MS medium supplemented with 7.5 mgL^{-1} BAP + 0.5 mgL^{-1} NAA at 3rd subculture

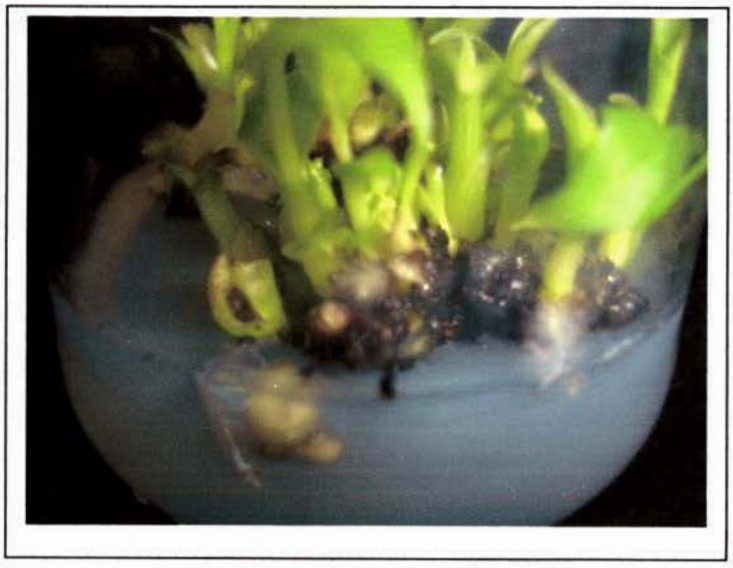

Plate 9. Multiple shoots of banana cv. BARI-1 produced on MS medium supplemented with 7.5 mgL^{-1} BAP + 0.5 mgL^{-1} NAA at 4th subculture

4.1.1.2 Length of shoot

The MS medium supplemented with BAP and NAA showed different results for increasing shoot length which was significantly influenced by different concentrations. The effect of different concentrations of NAA and BAP on the shoot length of banana cv. BARI-1 have been presented in Table 2. The longest shoot was produced by the treatment concentration 7.5 mgL^{-1} BAP + 0.5 mgL^{-1} NAA treatment (1.03, 2.45 and 3.38 cm) at 10, 20 and 30 DAI respectively. Statistically identical shoot length was observed in 5.0 mgL^{-1} BAP + 0.5 mgL^{-1} NAA at 20 DAI (2.43 cm) and 30 DAI (3.13 cm).

Rahaman *et al.* (2004) observed the similar result. They obtained longest shoot in the treatment 5.0 mgL^{-1} BAP (3.62 cm) followed by 1.5 mgL^{-1} NAA and 4.0 mgL^{-1} BAP (3.40 cm) using BARI Banana 1. They also found shortest leaves in 2.0 mgL^{-1} BAP. The shorter shoot length was produced by the control treatment (1.05 cm) where growth hormones were absent. 2.5 mgL^{-1} BAP + 2.0 mgL^{-1} NAA (1.50 cm) and 7.5 mgL^{-1} BAP (1.25 cm) treatments produced shorter shoot length closer to control treatments.

The results of present experiment agree with the findings of Khanam *et al.* (1996) who obtained longest shoot in banana on MS medium supplemented with 25 µM BAP treatments.

Table 2. Effect of different concentrations of BAP and NAA on mean shoot length of banana plantlet cv. BARI-1 at different days after inoculation

Treatments		Shoots length (cm)		
BAP (mgL^{-1})	NAA (mgL^{-1})	10 DAI	20 DAI	30 DAI
0	0	0.00 i	0.00 j	0.00 j
	0.5	0.30 gh	1.03 ghi	1.88 defgh
	1.0	0.30 gh	1.28 defgh	1.95 bcdefg
	1.5	0.23 h	1.68 bcde	2.33 b
	2.0	0.20 h	1.40 cdefg	1.78 fgh
2.5	0	0.23 h	1.23 efgh	1.90 cdefg
	0.5	0.30 gh	1.48 cdefg	2.08 bcdefg
	1.0	0.45 feg	1.28 defgh	1.80 efgh
	1.5	0.35 fgh	1.10 fghi	1.83 efgh
	2.0	0.45 efg	0.70 i	1.50 hi
5.0	0	0.25 h	0.88 hi	2.28 bc
	0.5	0.48 ef	2.43 a	3.13 a
	1.0	0.88 abc	2.05 ab	2.13 bcdef
	1.5	0.58 de	1.53 cdef	1.78 fgh
	2.0	0.55 e	1.28 defgh	1.70 gh
7.5	0	0.58 de	1.20 efgh	1.25 i
	0.5	1.03 a	2.45 a	3.38 a
	1.0	0.95 ab	1.75 bcd	2.23 bcd
	1.5	0.88 abc	1.88 bc	2.10 bcdef
	2.0	0.78 c	1.30 defgh	2.05 bcdefg
10.0	0	0.75 c	1.05 fghi	1.93 cdefg
	0.5	0.73 cd	1.60 bcde	2.18 bcde
	1.0	0.80 bc	1.48 cdefg	2.28 bc
	1.5	0.73 cd	1.35 defgh	1.98 bcdefg
	2.0	0.73 cd	1.23 efgh	1.90 cdefg
LSD value (0.01)		0.16	0.50	0.39
CV (%)		16.06	19.27	10.49

4.1.1.3 Leaf number per explant

The effect of different concentration of NAA and BAP has been presented in Table 3. The results showed that the maximum number of leaves (2.50, 3.25 and 7.00 leaves/explant at 10, 20 and 30 DAI respectively) produced on the medium supplemented with 7.5 mgL^{-1} BAP and 0.50 mgL^{-1} NAA. The second highest number of leaves (2.75, 4.00 and 6.75 leaves/explant at 10, 20 and 30 DAI respectively) produced on the medium supplemented with 5.0 mgL^{-1} BAP and 1.0 mgL^{-1} NAA. Rahman *et al.* (2004) found in the experiment of BAP and NAA combination that the maximum number of leaves (3.12/plantlet) at 30 DAI produced with 5.0 mgL^{-1} BAP which was identical with the treatment of 4.0 mgL^{-1} BAP + 1.50 mgL^{-1} NAA. Rabbani *et al.* (1996) obtained same results from 5.0 mgL^{-1} BAP. The lowest number of leaves (0.00, 0.50 and 2.25 at 10, 20 and 30 DAI leaves/explant) obtained from control treatment (Table 3) which is not agreed with the finding of Rabbani *et al.* (1996) and Rahman *et al.* (2004).

Table 3. Effect of different concentrations of BAP and NAA on leaf number of banana plantlet cv. BARI-1 at different days after inoculation

Treatments		Number of leaves		
BAP (mgL^{-1})	NAA (mgL^{-1})	10 DAI	20 DAI	**30 DAI**
0	0	0.00 e	0.5 c	2.25 d
	0.5	1.50 cd	2.25 b	4.75 c
	1.0	1.75 bcd	2.75 ab	4.50 c
	1.5	1.75 bcd	3.00 ab	5.50 bc
	2.0	1.50 cd	2.25 b	5.50 bc
2.5	0	1.50 cd	2.50 b	4.75 c
	0.5	1.75 bcd	3.00 ab	5.00 c
	1.0	2.25 abc	3.25 ab	5.50 bc
	1.5	1.50 cd	2.75 ab	5.75 abc
	2.0	2.00 abcd	3.25 ab	5.50 bc
5.0	0	1.25 d	2.5 b	4.75 c
	0.5	2.50 ab	3.25 ab	5.25 c
	1.0	2.75 a	4.00 a	6.75 ab
	1.5	2.25 abc	3.25 ab	5.50 bc
	2.0	1.75 bcd	2.75 ab	5.25 c
7.5	0	2.00 abcd	3.25 ab	5.25 c
	0.5	2.5 ab	3.25 ab	7.00 a
	1.0	1.75 bcd	2.75 ab	5.25 c
	1.5	2.00 abcd	3.00 ab	5.75 abc
	2.0	2.00 abcd	2.5 b	4.50 c
10.0	0	1.50 cd	3.25 ab	4.75 c
	0.5	2.00 abcd	2.5 b	4.50 c
	1.0	1.50 cd	3.25 ab	5.25 c
	1.5	2.25 abc	2.75 ab	4.75 c
	2.0	1.75 bcd	2.75 ab	4.75 c
LSD value (0.01)		0.97	1.28	1.39
CV (%)		28.71	24.22	14.46

4.1.1.4 Length of largest leaves

The MS medium supplemented with BAP and NAA showed different results for increasing shoot length which was significantly influenced by different concentrations. The effect of different concentration of BAP and NAA on the length of largest leaves of banana cv. BARI-1 has been presented in Table 4. The longest leaves was produced by the treatment concentration 7.5 mgL^{-1} BAP + 0.5 mgL^{-1} NAA treatment (0.85, 2.70 and 4.23 cm at 10, 20 and 30 DAI respectively) which was statistically significant. Statistically

identical leaves length was observed in 7.5 mgL^{-1} BAP + 1.0 mgL^{-1} NAA at 20 DAI (2.10 cm) and 30 DAI (3.70 cm).

Rahaman *et al.* (2004) observed the similar result. They obtained longest leaves in the treatment 5.0 mgL^{-1} BAP (3.62 cm) followed by 1.5 mgL^{-1} NAA and 4.0 mgL^{-1} BAP (3.40 cm) using BARI Banana 1. They also found shortest leaves in 2.0 mgL^{-1} BAP. The shorter leaves length was produced by the control treatment (0.95 cm) where growth hormones were absent.

The results of present experiment agree with the findings of Khanam *et al.* (1996) who obtained longest leaves in banana on MS medium supplemented with 25 µM BAP treatments.

Table 4. Effect of different concentrations of BAP and NAA on length largest leaves of banana plantlet cv. BARI-1 at different days after inoculation

Treatments		Length of largest leaves (cm)		
BAP (mgL^{-1})	NAA (mgL^{-1})	10 DAI	20 DAI	30 DAI
0	0	0.50 defg	0.55 h	0.95 i
	0.5	0.58 cdef	1.65 fg	2.03 gh
	1.0	0.60 bcde	1.70 efg	1.95 h
	1.5	0.70 abc	1.80 bcdefg	2.00 gh
	2.0	0.46 defg	1.60 g	2.08 gh
2.5	0	0.50 defg	1.55 g	1.95 h
	0.5	0.63 bcd	2.03 bcd	2.58 efg
	1.0	0.600 bcde	1.95 bcdef	2.53 efgh
	1.5	0.45 efg	2.075 bc	2.43 fgh
	2.0	0.45 efg	2.05 bcd	2.28 gh
5.0	0	0.35 g	1.68 fg	2.15 gh
	0.5	0.75 ab	2.60 a	3.60 bc
	1.0	0.70 abc	2.05 bcd	3.10 cde
	1.5	0.50 defg	2.03 bcd	2.93 def
	2.0	0.50 defg	2.00 bcde	2.98 def
7.5	0	0.43 fg	1.65 fg	2.23 gh
	0.5	0.85 a	2.70 a	4.23 a
	1.0	0.70 abc	2.10 b	3.70 ab
	1.5	0.55 cdef	1.95 bcdef	2.58 efg
	2.0	0.55 cdef	1.76 cdefg	2.28 gh
10.0	0	0.53 def	1.53 g	2.15 gh
	0.5	0.50 defg	1.75 defg	2.98 def
	1.0	0.43 fg	1.93 bcdef	3.23 bcd
	1.5	0.45 efg	1.95 bcdef	3.23 bcd
	2.0	0.45 efg	1.60 g	3.08 cde
LSD value (0.01)		0.16	0.31	0.58
CV (%)		15.12	8.86	11.86

4.1.2 Experiment 2.Effect of IAA and IBA on root proliferation of banana cv. BARI1.

The regenerated shoots were collected from *in vitro* grown plants in experiment 1. Then it was subcultured on half strength MS medium supplemented with different levels of IBA (0, 0.5, 1.0 and 1.50 mgL^{-1}) and IAA (0, 0.5 and 1.0 mgL^{-1}) in order to allow root formation. Root numbers varied with different concentrations of IBA and IAA. The results on the effect of different concentration of IBA and IAA on root formation have been discussed with following headings.

4.1.2.1 Number of root per explant

The effect of IAA and IBA on the number of root per explant produced by different combination at 10, 20 and 30 DAI was shown in the Table 5 showed significant variation. The highest number of root was produced by 0.5 mgL^{-1} IAA + 0.5 mgL^{-1} IBA (3.50, 4.50 and 6.50 per explant respectively) which was statistically significant than other treatment (Table 5). 0.5 mgL^{-1} IAA + 1.0 mgL^{-1} IBA produced 6.0 roots per explant at 30 DAI but at 20 DAI 3.50 roots was produced per explant. The lowest number of root was produced by control treatment. Vigorous roots of *in vitro* grown plantlet on MS media supplemented with 0.5 mgL^{-1} IAA + 0.5 mgL^{-1} IBA was shown in plate 10 (a & b). The present results are similar with the findings of Gubbuk and Pekmezci (2001).

Molla *et al.* (2004) obtained 8.28 number of roots/plantlet on 0.5 mgL^{-1} IBA followed by 6.33 roots, 0.6 mgL^{-1} BA. They also observed 3.89 and 3.97 number of roots in 0.2 mgL^{-1} IBA and 0.3 mgL^{-1} IBA respectively. Present results in similar of Molla *et al.* (2004) results.

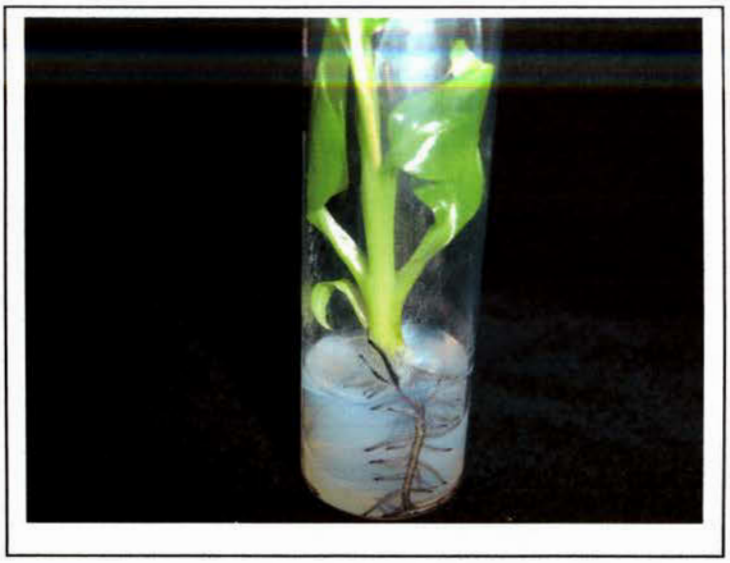

Plate 10. a & b. Vigorous roots of banana cv. BARI-1 grown on MS media supplemented with 0.5 mgL^{-1} IAA + 0.5 mgL^{-1} IBA

Table 5. Effect of different concentrations of IAA and IBA on root number of multiplied shoot of banana cv. BARI-1 at different days after inoculation

Treatments		Vigour of regenerated root	Number of root		
IAA (mgL^{-1})	IBA (mgL^{-1})		10 DAI	20 DAI	30 DAI
0	0	+	0.00 e	0.00 e	0.00 f
	0.5	+	1.50 cd	2.00 d	3.25 e
	1.0	++	2.25 bc	2.25 d	3.50 de
	1.5	++	2.25 bc	2.50 cd	3.50 de
0.5	0	+++	2.75 ab	2.25 d	3.25 e
	0.5	+++	3.50 a	4.50 a	6.50 a
	1.0	++	3.25 ab	3.50 abc	6.00 ab
	1.5	++	3.25 ab	4.00 ab	5.00 bc
1.0	0	+	1.00 de	2.50 d	3.25 e
	0.5	+	2.75 ab	2.75 cd	3.75 de
	1.0	++	1.50 cd	3.00 bcd	4.00 cde
	1.5	+	1.25 cd	3.50 abc	4.50 cd
LSD value (0.01)			1.03	1.16	1.16
CV (%)			25.36	22.19	15.51

\+ = Less vigorous growth
++ = Good growth and vigour
+++ = Best growth and vigour

The results of the present experiment similar with the findings of Khanam *et al.* (1996). Rahman *et al.* (2004) obtained highest roots (2.88/explant) at 30 DAI by 3.0 mgL^{-1} NAA where as not respond on control.

4.1.2.2 Length of root

The length of roots developed by the plantlets was influenced considerably by different concentration of IAA and IBA used in the experiments and the results have been presented in Table 6.

Table 6. Effect of different concentrations of IAA and IBA on root length of multiplied shoot of banana cv. BARI-1 at different days after inoculation

Treatments		Root length (cm)		
IAA (mgL^{-1})	IBA (mgL^{-1})	10 DAI	20 DAI	30 DAI
0	0	0.00 e	2.00 ef	2.00 f
	0.5	1.08 d	1.88 f	2.30 e
	1.0	1.08 d	2.30 de	3.15 d
	1.5	1.13 d	2.45 d	3.08 d
0.5	0	1.23 d	1.60 f	2.08 e
	0.5	2.93 a	4.63 a	5.88 a
	1.0	2.55 ab	3.88 b	4.83 b
	1.5	3.03 a	3.85 b	4.88 b
1.0	0	1.80 c	2.33 de	3.45 cd
	0.5	1.80 c	2.35 de	3.48 cd
	1.0	2.08 bc	3.15 c	3.75 c
	1.5	2.15 bc	2.70 d	3.70 c
LSD value (0.01)		0.49	0.42	0.55
CV (%)		16.28	7.95	7.57

The result indicated that there was a sharpe increasing trends in root length at different DAI (10, 20 and 30 DAI) which is significant at 1% level.

The root length of plantlets after 30 DAI was remarkably highest which is Auxin was essential for successful root induction of banana and has been reported by Raut and Lokhand (1989).

Plate 11. Well established meristern derives plantlets Banana BARI-1 in poly bags.

The highest length was observed at 10, 20 and 30 DAI in the treatment concentration 0.5 mgL^{-1} IAA and IBA (2.93, 4.63 and 5.88 cm) which was statistically significant.

The second highest result (3.03, 3.85 and 4.88 cm at 10, 20 and 30 DAI) was observed with 0.5 mgL^{-1} IAA and 1.5 mgL^{-1} IBA and the lowest (0.00, 2.00 and 2.00 cm at 10, 20 and 30 DAI) value obtained with control treatment. Similar results was obtained by Molla *et al.* (2004) where they got 2.60-5.67 cm range of root length in 0.5 mgL^{-1} IBA.

Habiba (2002), Khanam *et al.* (1996) and Ali (1996) also got more or less same observation. Therefore, the presented result partially agrees with the findings of Gubbuk and Pekmezci (2001) who reported that 1.0 µM IBA per litre with MS medium.

4.2 Established Plantlet

Meristem derived plantlet transferred to poly bags containing 1:1 (ground soil : cowdung) mixture after 7 days hardening in room temperature (28-30°C). A good established plantlet were shown in plate 11, which is ready for planting.

CHAPTER V

SUMMARY WITH FUTURE SUGGESTIONS

The experiments were conducted at the Biotechnology Laboratory, Bangladesh Agricultural Research Institute, Gazipur, during the period from September 2004 to June 2005 to investigate the effect of different concentrations of BAP, NAA, IAA and IBA on regeneration, on shoot proliferation and root formation in Banana (*Musa* spp). Meristems were collected from about four months old banana suckers were used on explants.

In experiment 1, BAP (0.0, 2.5, 5.0, 7.5 and 10.0 mgL^{-1}) and NAA (0.0, 0.5, 1.0, 1.5 and 2.0 mgL^{-1}) were used as treatments.

The meristem was excised under a sterio binocular microscope and transferred to solid meida. *In vitro* culture of meristem results hard meristematic ball like structure in regeneration media containing different concentration of BAP and NAA. The culture meristem first turned brown in colour in 4-5 days and after 30-50 days later a green globular hard coat mass grow to be round in shape producing a ball like structure. From this ball like structure adventitious plantlets were developing.

The number of shoots produced per explant was varied in MS media supplemented with different concentrations of BAP and NAA. Data were recorded at 10, 20 and 30 Days After Inoculation (DAI).

Among the different concentration 7.5 mgL^{-1} BAP + 0.5 mgL^{-1} NAA showed highest shoot proliferation 0.75, 2.75 and 6.25 shoots per explant at 10, 20 and 30

DAI respectively. Followed by 7.5 mgL^{-1} BAP + 1.0 mgL^{-1} NAA treatment (0.75, 2.75 and 5.25 shoots per explant at 10, 20 and 30 DAI respectively. A good number of shoot proliferation was achieved at 7.5 mgL^{-1} BAP + 1.5 mgL^{-1} NAA at 30 DAI (4.25) which is superior from the control treatments (1.00). The longest shoot was produced by the treatment concentration 7.5 mgL^{-1} BAP + 0.5 mgL^{-1} NAA treatment (1.03, 2.45 and 3.38 cm) at 10, 20 and 30 DAI respectively. Statistically identical shoot length was observed in 5.0 mgL^{-1} BAP + 0.5 mgL^{-1} NAA at 20 DAI (2.43 cm) and 30 DAI (3.13 cm). The maximum number of leaves (2.50, 3.25 and 7.00 leaves/explant at 10, 20 and 30 DAI) produced on the medium supplemented with 7.5 mgL^{-1} BAP and 0.50 mgL^{-1} NAA. The second highest number of leaves (2.75, 4.00 and 6.75 leaves/explant at 10, 20 and 30 DAI) produced on the medium supplemented with 5.0 mgL^{-1} BAP and 1.0 mgL^{-1} NAA. The longest leaves was produced by the treatment concentration 7.5 mgL^{-1} BAP + 0.5 mgL^{-1} NAA treatment (0.85, 2.70 and 4.23 cm at 10, 20 and 30 DAI) respectively which was statistically significant. Statistically identical leaves length was observed in 7.5 mgL^{-1} BAP + 1.0 mgL^{-1} NAA at 20 DAI (2.10 cm) and 30 DAI (3.70 cm).

The regenerated shoots were collected from *in vitro* grown plants in experiment 1. Then it was subcultured on half strength MS medium supplemented with different levels of IBA (0, 0.5, 1.0 and 1.50 mgL^{-1}) and IAA (0, 0.5 and 1.0 mgL^{-1}) in order to allow root formation. Root numbers varied with different concentrations of IBA and IAA.

The highest number of root was produced by 0.5 mgL^{-1} IAA + 0.5 mgL^{-1} IBA (3.50, 4.50 and 6.50 per explant respectively), which was statistically significant than other treatment (Table 5). 0.5 mgL^{-1} IAA + 1.0 mgL^{-1} IBA produced 6.0 roots per

explant at 30 DAI but at 20 DAI 3.50 roots was produced per explant. The lowest number of root was produced by control treatment. The highest length was observed at 10, 20 and 30 DAI in the treatment concentrations 0.5 mgL^{-1} IAA and IBA (2.93, 4.63 and 5.88 cm respectively) which was statistically significant. The second highest result (3.03, 3.85 and 4.88 cm at 10, 20 and 30 DAI respectively) was observed with 0.5 mgL^{-1} IAA and 1.5 mgL^{-1} IBA and the lowest (0.00, 2.00 and 2.00 cm at 10, 20 and 30 DAI respectively) value obtained with control treatment. Meristem derived plantlet transferred to poly bags containing 1:1 (ground soil : cowdung) mixture after 7 days hardening in room temperature (28-30°C). A good established plantlet which is ready for planting.

Meristem derived plantlet micro-propagation protocol is well developed. In future virus free planting material should be conformed by using ELISA Test method for each step of plantlet production.

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Appendix i. Compositions and concentrations used for the preparation of MS media (Murashige and Skoog, 1962)

No.	Compositions	Concentration (g/l)
	Micronutrient (10 X)	
1.	KNO_3	19.0
2.	NH_4NO_3	16.50
3.	KH_2PO_4	1.70
4.	$CaCl_2.2H_2O$	4.40
5.	$MgSO_4.7\ H_2O$	3.70

No.	Micronutrient (100 X)	Concentration (mg/l)
1.	$MnSO_4.\ 4H_2O$	2230.0
2.	H_3BO_3	620.0
3.	$ZnSO_4.7H_2O$	860.0
4.	KI	83.0
5.	$CuSO_4.5H_2O$	2.5
6.	$Na_2M_oO_4.2H_2O$	25.0
7.	$CoCl_2.\ 6H_2O$	2.5

Fe-EDTA source (100 X)	Concentration (mg/l)
$FeSO_4.\ 7H_2O$	2.78
$Na_2EDTA.\ 2H_2O$	3.73

No.	Organic supplement (100 X)	Amount per liter (mg)
1.	Myo-Inositol	10000.0
2.	Glycine	200.0
3.	Nicotinic acid	50.0
4.	Pyridoxine HCl	50.0
5.	Thiamine HCl	10.0
	Sucrose	30 g
	Agar	7 g

Appendix ii. Analysis of variance on No. of shoots 10 DAI

	Degrees of freedom	Sum of squares	Mean square	F-value	Probability
Between	24	5.440	0.227	2.125	0.0072
Within	75	8.000	0.107		
Total	99	13.440			

Coefficient of variation = 38.88%

Appendix iii. Analysis of variance on No. of shoots 10 DAI on no. of shoots 20 DAI

	Degrees of freedom	Sum of squares	Mean square	F-value	Probability
Between	24	20.060	0.836	2.668	0.0007
Within	75	23.500	0.313		
Total	99	43.560			

Coefficient of variation = 34.55%

Appendix iv. Analysis of variance on No. of shoots 30 DAI

	Degrees of freedom	Sum of squares	Mean square	F-value	Probability
Between	24	130.660	5.444	11.922	0.0000
Within	75	34.250	0.457		
Total	99	164.910			

Coefficient of variation = 26.71%

Appendix v. Analysis of variance on shoot length (cm) 10 DAI

	Degrees of freedom	Sum of squares	Mean square	F-value	Probability
Between	24	7.276	0.303	40.600	0.0000
Within	75	0.560	0.007		
Total	99	7.836			

Coefficient of variation = 16.06%

Appendix vi. Analysis of variance on shoot length (cm) 20 DAI

	Degrees of freedom	Sum of squares	Mean square	F-value	Probability
Between	24	25.194	1.050	14.778	0.0000
Within	75	5.327	0.071		
Total	99	30.521			

Coefficient of variation = 19.27%

Appendix vii. Analysis of variance on shoot length (cm) 30 DAI

	Degrees of freedom	Sum of squares	Mean square	F-value	Probability
Between	24	39.435	1.643	37.773	0.0000
Within	75	3.262	0.043		
Total	99	42.698			

Coefficient of variation = 10.49%

Appendix viii. Analysis of variance on No. of leaves 10 DAI

	Degrees of freedom	Sum of squares	Mean square	F-value	Probability
Between	24	27.140	1.131	4.188	0.0000
Within	75	20.250	0.270		
Total	99	47.390			

Coefficient of variation = 28.71%

Appendix ix. Analysis of variance on No. of leaves 20 DAI

	Degrees of freedom	Sum of squares	Mean square	F-value	Probability
Between	24	37.760	1.573	3.371	0.0000
Within	75	35.000	0.467		
Total	99	72.760			

Coefficient of variation = 24.22%

Appendix x. Analysis of variance on No. of leaves 30 DAI

	Degrees of freedom	Sum of squares	Mean square	F-value	Probability
Between	24	72.060	3.003	5.459	0.0000
Within	75	41.250	0.550		
Total	99	113.310			

Coefficient of variation = 14.46%

Appendix xi. Analysis of variance on Length of largest leaves (cm) 10 DAI

	Degrees of freedom	Sum of squares	Mean square	F-value	Probability
Between	24	1.355	0.056	8.220	0.0000
Within	75	0.515	0.007		
Total	99	1.870			

Coefficient of variation = 15.12%

Appendix xii. Analysis of variance on Length of largest leaves (cm) 20 DAI

	Degrees of freedom	Sum of squares	Mean square	F-value	Probability
Between	24	15.037	0.627	23.350	0.0000
Within	75	2.012	0.027		
Total	99	17.050			

Coefficient of variation = 8.86%

Appendix xiii. Analysis of variance on Length of largest leaves (cm) 30 DAI

	Degrees of freedom	Sum of squares	Mean square	F-value	Probability
Between	24	47.286	1.970	20.609	0.0000
Within	75	7.170	0.096		
Total	99	54.456			

Coefficient of variation = 11.86%

Appendix xiv. Analysis of variance on No. of roots 10 DAI

	Degrees of freedom	Sum of squares	Mean square	F-value	Probability
Between	11	50.229	4.566	16.038	0.0000
Within	36	10.250	0.285		
Total	47	60.479			

Coefficient of variation = 25.36%

Appendix xv. Analysis of variance on No. of roots 20 DAI

	Degrees of freedom	Sum of squares	Mean square	F-value	Probability
Between	11	58.917	5.356	14.832	0.0000
Within	36	13.000	0.361		
Total	47	71.917			

Coefficient of variation = 22.19%

Appendix xvi. Analysis of variance on No. of roots 30 DAI

	Degrees of freedom	Sum of squares	Mean square	F-value	Probability
Between	11	118.250	10.780	29.769	0.0000
Within	36	13.000	0.361		
Total	47	131.250			

Coefficient of variation = 15.51%

Appendix xvii. Analysis of variance on Length of roots 10 DAI

	Degrees of freedom	Sum of squares	Mean square	F-value	Probability
Between	11	34.217	3.111	38.985	0.0000
Within	36	2.873	0.080		
Total	47	37.090			

Coefficient of variation = 16.28%

Appendix xviii. Analysis of variance on Length of roots 20 DAI

	Degrees of freedom	Sum of squares	Mean square	F-value	Probability
Between	11	37.747	3.432	71.407	0.0000
Within	36	1.730	0.048		
Total	47	39.477			

Coefficient of variation = 7.95%

Appendix xix. Analysis of variance on Length of roots 30 DAI

	Degrees of freedom	Sum of squares	Mean square	F-value	Probability
Between	11	100.964	9.179	140.309	0.0000
Within	36	2.355	0.065		
Total	47	103.319			

Coefficient of variation = 7.57%