# *IN VITRO* REGENERATION OF BANANA CV. AMRITASAGAR AND SABRI THROUGH SHOOT TIP CULTURE


BY

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REGISTRATION NO. 03-01145


A Thesis
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**

**GENETICS AND PLANT BREEDING**

SEMESTER: JULY-DECEMBER, 2008

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# *CERTIFICATE*

This is to certify that thesis entitled, ***"IN VITRO* REGENERATION OF BANANA CV. AMRITASAGAR AND SABRI THROUGH SHOOT TIP CULTURE"** 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 GENETICS AND PLANT BREEDING**, embodies the result of a piece of bona fide research work carried out by **MD. FERDOUS HOWLADER**, Registration No. 03-01145 under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.

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

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**Dated: December, 2008**
**Place: Dhaka, Bangladesh**

**(Dr. Md. Shahidur Rashid Bhuiyan)**
**Professor**
**Supervisor**

*Dedicated*

*to*

*My Beloved Parents*

## SOME COMMONLY USED ABBREVIATIONS AND SYMBOLS

| Abbreviations | Full word |
|---|---|
| °C | : Degree Celsius |
| % | : Percent |
| µM | : Micro mole |
| 2, 4-D | : 2, 4-dichlorophenoxy acetic acid |
| Agric. | : Agriculture |
| Agril. | : Agricultural |
| BAP | : 6-benzyl amino purine |
| BBS | : Bangladesh Bureau of Statistics |
| cm | : Centimeter |
| Contd. | : Continued |
| CRD | : Completely Randomized Design |
| cv. | : Cultivar |
| DAI | : Days After Inoculation |
| dw | : Distilled Water |
| *et al.* | : And others |
| etc. | : Etcetera |
| FAO | : Food and Agricultural Organization |
| g | : Gram |
| g/l | : Gram per litre |
| ha | : Hectare |
| $ha^{-1}$ | : Per hectare |
| hr. | : Hour(s) |
| i.e. | : id est (That is) |
| IBA | : Indole-3-butyric acid |
| Int. | : International |
| J. | : Journal |
| mg | : Milligram(s) |
| mg/l | : Milligram per litre |
| ml | : Milliliter(s) |
| MS | : Murashige and Skoog |
| NaOH | : Sodium Hydroxide |
| No. | : Number |
| NS | : Not significant |
| pH | : Negative logarithm of hydrogen ion concentration (-log $[H^+]$) |
| SAU | : Sher-e-Bangla Agricultural University |
| Sci. | : Science |
| Univ. | : University |
| Viz | : Namely |
| w/v | : Weight/volume |

# ACKNOWLEDGEMENT

*First of all the author expresses his gratitude to the merciful and almighty Allah without Whose grace he would have never been able to pursue his higher studies in this field of science and to complete this thesis for the degree of MS in Genetics and Plant Breeding.*

*The author expresses his ever indebtedness, deepest sense of gratitude, sincere appreciation and profound regards to his reverend and beloved teacher and Research Supervisor Dr. Md. Shahidur Rashid Bhuiyan, Professor, Department of Genetics and Plant Breeding, Sher-e-Bangla Agricultural University, Dhaka for his scholastic supervision, uncompromising principles, valuable advices, constant inspiration, radical investigation and constructive criticism in all phases of this study and preparing the manuscript.*

*The author finds it a great pleasure in expressing his heartfelt gratitude and immense indebtedness to his honorable and respected teacher and Research Co-Supervisor, Dr. Ather-uz-Zaman, Head of Operation, Proshika Tissue Culture Centre Trust, Manikgonj for his kind co-operation, creative suggestions, continuous encouragement and constructive criticism for the completion of the research work and preparation of the thesis.*

*The author expresses his deepest respect and boundless gratitude to Dr. Firoz mahmud, Associate Professor and Chairman, Department of Genetics and Plant Breeding, Sher-e-Bangla Agricultural University, Dhaka for his sympathetic co-operation and inspiration throughout the research work.*

*The author is thankful to Professor Mahammad Hossain Bhuiyan, Co-ordinator (CASR) for providing necessary facilities and conductive atmosphere to accomplish his research work.*

*Special thanks and indebtedness are also due to all the respective teachers of the Department of Genetics and Plant Breeding, Sher-e-Bangla Agricultural University, Dhaka for their valuable teaching, sympathetic co-operation and inspiration throughout the period of the study.*

*The author also likes to give thanks to all of his friends for their support and inspiration throughout his study period in SAU, Dhaka.*

*Finally, the author found no words to thank his parents, his brother and sister for their unquantifiable love and continuous support, their sacrifice never ending affection, immense strength and untiring efforts for bringing his dream to proper shape. They were constant source of inspiration, zeal and enthusiasm in the critical moment of his studies.*

*Dated: December, 2008*
*Place: SAU, Dhaka*

*The Author*

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# *IN VITRO* REGENERATION OF BANANA CV. AMRITASAGAR AND SABRI THROUGH SHOOT TIP CULTURE

## ABSTRACT

BY
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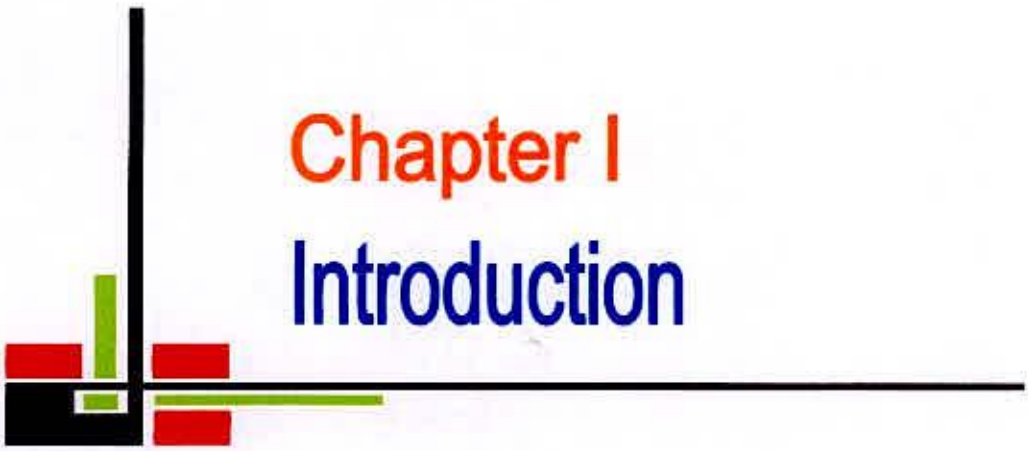
The study was undertaken with a view to establish a protocol for *in vitro* culture and plant regeneration from shoot tip explants of banana cv. Amritasagar and Sabri. This experiment was setup at Proshika Tissue Culture Centre Trust (PTCCT) laboratory, Manikgonj during the period from April to November, 2008. In the present study, BAP (0.0, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0 and 7.0 mg/l) and IBA (0.0, 1.0, 2.0, 3.0, 4.0 and 5.0 mg/l) have been used in different concentrations on MS medium to assess the influence on *in vitro* shoot regeneration and subsequent root formation. Explants were sterilized by 0.1% $HgCl_2$ for 14 minutes and cent percent shoot tip explants were found free from contamination after 10 days of sterilization and inoculation. Explants of Amritasagar and Sabri turned brown, light green, green and dark green in colour after 10 days of inoculation but after 20 days all became dark green at all concentrations of BAP except control in Amritasagar and control and 1.0 mg/l in Sabri. The response of single shoot formation in Amritasagar was the highest (50%) followed by Sabri (30%) at 5.0 mg/l BAP within 10-15 days. Among the concentrations of BAP, 5.0 mg/l showed the best shoot proliferation and the lowest was at 1.0 mg/l. No response was observed at control. Shoot proliferation rate of Amritasagar was better than that of Sabri. The cultivar Amritasagar and Sabri produced 3.50 shoots/explant and 2.00 shoots/explant, respectively at 5.0 mg/l BAP at 30 DAI. Amritasagar also produced longer shoot (2.64 cm) compared to Sabri (2.16 cm) at 5.0 mg/l BAP at 30 DAI. Among the concentrations of IBA, 3.0 mg/l proved to be the best for root induction. Amritasagar showed superior performance (4.10 roots/plantlet) for root induction over Sabri (3.63 roots/plantlet) at 3.0 mg/l IBA at 30 DAI. The survival of the plantlets of both cultivars under *ex vitro* condition was more than 82% and all of them resumed growth in the field.



# Chapter I

# Introduction

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# INTRODUCTION

Banana (*Musa* spp.) belonging to the family Musaceae is the most ancient fruit plant having been used and cultivated in over 100 countries throughout the tropical and subtropical countries. Today, it is considered as the fourth most important global food commodity after rice, wheat and maize in terms of the gross value of production (FAO, 2002). The largest banana producing regions in the world are Africa and Latin America, which represent 74.2 and 22.5 percent of the world production, respectively followed by Asia with 3.3% (Rodriguez, 2001). It is the only fruit which remains available throughout the year and its consumption rate is also higher than other fruits. It is a rich source of vitamins, minerals and carbohydrates, which are assimilated easily and provide energy (104 cal/100g) for human body (Simmonds, 1959). Bananas are a valuable source of vitamin A, vitamin $B_6$, vitamin C and potassium.

In our country, Banana is regarded as the most important starch rich horticultural crop. In Bangladesh bananas are grown all over the country and the cultivar Amritasagar is very popular and grown widely in the greater district of Jessore, Kustia, Dhaka, Rajshahi, Mymensingh, Rangpur and Dinajpur.

Bangladesh produced about 877.12 thousand metric tones of banana from 131.63 thousand acres of land (BBS, 2008). This yield is quite low compare to other banana growing countries of the world like Argentina (34 t/ha) and Costa Rica (33 t/ha) (FAO, 2002). It is due to diseases and lack of high yielding varieties. Moreover, every year a large number of banana crop fields are destroyed due to flood or heavy rainfall. The banana cultivars generally growing in Bangladesh are Amritasagar, Sabri, Champa, Chinechampa, Kabri,

Kabuli, Jahaji, Agniswar, Basrai, seeded Banana, Anaji or Kachakola etc. (Haque, 1988). Among the cultivars, Amritasagar and Sabri are considered as the best quality banana of Bangladesh. They are seedless, delicious, possesses good colour and aroma when ripen.

*Musa* spp. are clonally propagated, grown from massive underground corm with highly compressed internodes and are subjected to attack by a wide range of diseases and pests (Cronauer and Krikorian, 1983) which carries over through generations and affect the yield. Thus, the productivity of fruits decreases and finally the yield becomes very poor and static which affects the normal economy. To minimize the above mentioned problems, disease free plant material should be produced and maintained, so that it could retain its good production capacity. This can be possible by meristem culture, because the concentration of pathogen gradually decreases at the approach of the shoot tips and finally absent in the meristem.

The traditional methods for propagation of *Musa* spp. are laborious, time consuming and not very efficient as far as production of large number of homogenous plant is concerned (Banerjee *et. al.*, 1987). Moreover, the rate of propagation by suckers in this method is rather low (5 to 10 suckers/year) whereas in tissue culture method, over a million of plants can be grown from a small or even a microscopic piece of plant tissue within a year. In terms of 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 plants of banana performed better than the conventional method of propagation through sword suckers (Faisal *et al.*, 1998).

The application of classical methods of breeding for both disease and pest resistance has resulted in only limited success due to the long generation times for banana and the high sterility and triploidy of most cultivated bananas

(Sasson, 1997). The integration of genetic engineering into breeding programs may provide powerful tools to overcome these limitations by introducing specific changes that can be utilized for banana improvement within a short period of time. However, these applications require reliable plant regeneration protocols for banana.

Banana tissue culture is a first generation plant biotechnology tool used to reduce the spread of pests and diseases; especially fungal and bacterial wilt diseases, by making disease free planting material available for the propagation of new crops. Simple tissue culture techniques such as shoot-tip and embryo culture are well developed in Africa and have greatly improved banana breeding, whereby the shoot meristem is extracted from the male flower and aseptically multiplied into hundreds of shoot for eventual planting in fields.

Plant growth regulators play an important role for *in vitro* regeneration of any crop plants grown in any artificial medium. Generally cytokinin helps in shoot multiplication and auxin helps in rooting of multiplied shoots. However, the requirement of cytokinin and auxin depends on the variety and culture conditions (Cronauer and Krikorian, 1984a). Considering the above situations, the present study was undertaken with the following objectives-

I. To assess the suitable concentration of hormone for *in vitro* shoot proliferation,
II. To assess the suitable concentration of hormone for *in vitro* root induction, and
III. To establish a commercial protocol for the production of disease free healthy banana plantlets

# Chapter II

# Review of literature

# CHAPTER II

# REVIEW OF LITERATURE

Banana (*Musa* spp.) is one of the most important fruit in many tropical and subtropical countries including Bangladesh, which is quick growing fruit generally propagated by sucker. Advances in the techniques of micro propagation by tissue culture have revolutionized the propagation of bananas in the last 30 years. Researches on micro propagation of banana have been carried out in different countries of the world. The use of tissue culture enables farmers to plant with material certified as disease and pest free. In many situations this strategy can delay the requirement to use chemical control measures. There is a little detailed literature on the regeneration and production of specific pathogen free plantlets of various *Musa* clones by means of aseptic culture techniques in many countries of the world, whereas it is very limited in Bangladesh when banana is an important crop. The research findings on regeneration of banana related to present study have been reviewed in this chapter under different headings.

## 2.1 Regeneration of banana

Bhuiyan (2007) carried out an experiment on clonal propagation of banana cv. Amritasagar and BARI-1. The results showed that the best performance was observed in MS+5.0 mg/l BAP+2.0 mg/l KIN+2.0 mg/l IAA and MS+4.0 mg/l BAP+2.0 mg/l IBA+2.0 mg/IAA for shoot proliferation. It was observed that the number of multiple shoots per culture was maximized 15.80 and 12.50 respectively up to $7^{th}$ subculture but beyond that the rate of multiple shoot formation was declined considerably.

Khaldun (2005) reported that the shoot proliferation rate of Amritasagar and Sabri were 3.67 explant$^{-1}$ and 2.00 explant$^{-1}$, respectively after 30 days of culture initiation in 5.0 mg/l BAP. Longest shoots (3.53 cm) were achieved using 5.0 mg/l BAP in Amritasagar followed by 2.02 cm in Sabri at 30DAI.

Rahman (2003) conducted an experiment on the standardization of regeneration protocol of banana cv. BARI-1 through meristem culture, where 5.0 mg/l BAP produced the highest percentage (70.69) of single shoot with maximum shoot length (3.63 cm). The treatment 4.0 mg/l BAP+1.5 mg/l NAA produced the greatest number of shoots (4.52/explant) and the longest root (5.86 cm) was obtained from 2.0 mg/l IBA+1 g/l charcoal treatment.

Helliot *et al.* (2002) carried out an experiment on the utilization of cryopreservation for the eradication of Banana Streak Virus (BSV) from *Musa* spp. 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 Sagar was MS medium supplemented with 4.0 mg/l BAP and 1.0 mg/l KIN. Whereas, the best medium for shoot multiplication was MS medium fortified with 4.0 mg/l BAP, 2.0 mg/l IAA and 13% CW. Average time required for production of single shoot and multiple shoot were 15-21 days and 40-45 days, respectively.

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 mg/l and BAP 0.5 mg/l. In general, Kanchkala (*Musa,* ABB group) showed the best explant survival as well as growth response. Martaman *(Musa,* ABB group) also showed good growth but comparatively poor explant 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 micropropation of banana 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 paradashca* 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 with 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 cultured on semisolid MS medium supplemented with various growth regulators. For all the three types of explant, the shortest time taken for culture establishment (9-12 per explant) was obtained on medium supplemented with 10 mg/l BAP regardless of NAA concentration.

Ramos and Zanlora (1990) studied on elimination of banana bunchy top infection from banana *(Musa* sp. cv. Lacatan) by heat pretreatment and meristem culture, where they found that heat pre-treatment ($40^0$C for 16hr. photoperiod and $32^0$C for 8hr. darkness) daily for 1 and 2 months of *in vitro* shoot cultures of BBTV infected banana cv. Lakatan 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.

Besides shoot tip culture, callus induction, proliferation and slow growing cell suspension form mature fruits, leaf sheath primordia, rhizome tissue were also reported (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). Formation of spherical callus masses in *Musa* spp. resembling somatic embryo 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.

Rivas (1988) obtained virus free plants of banana cv. Payo and Giant Cavendish infected by Cucumber Mosaic Virus by meristem culture in a medium containing Murashige and Skoog salts supplemented with thiamin HCl (1 mg/l). Pyridoxin (1 mg/1), nicotinic acid (100 mg/1) and myo inositol (100 mg/1) solidified with 7 g/1 bactoagar.

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 the best achieved with the 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.

Wilson *et al.* (1987) found that the apical meristem (4 month old plants) is destroyed using a knife to cut an opening at the base of the pseudostem. This method help to retain the foliage of the mother plant produced about 70% more suckers 2 months earlier than the old method in which both foliage and apical meristem were destroyed. It is recommended where there are no facilities for meristem culture.

Hwang and Ko (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, pseudostern colour and bunch characters. Meristem derived plants were screened for resistance to *F. oxysporum* 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 semisolid 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 mg/l BAP.

Berg and Bustamante (1974) conducted an experiment on heat treatment and meristem culture for the production of virus-free bananas. Banana rhizomes were heat treated at 35-43$^{0}$C. Meristems from lateral buds that developed after 100 days of treatment were excised and grown on a modified Knudson's medium to which NAA was added to induce root formation. Virus could not be detected in plants derived from meristems by inoculations of indicator plants

*(Chertopodiuni quinoa* and *C. amaranticotor)*. They reported that localized or systemic symptoms were often induced when indicator plants were inoculated with leaf material from control plants is commercial plantations. Heat treatment of banana rhizomes or meristem culture alone is not enough to free bananas of viruses. When the two techniques were combined approximately 75% of the plants were found to be free of the symptomless viruses which can be detected by indicator plants studies are being initiated to determine what effect viruses have on plant vigour.

An investigation was carried out by Khatri *et al.* (1972) using meristematic tip with two pairs of leaf primordia from clones of dessert banana *(Musa* spp) viz GN60A, SH3362, William Highgate and Basrai were evaluated for *in vitro* propagation. They reported that clone GN60A produced highest tillers followed by clone SH3362, with lowest number of tillers produced by clone Basrai when the proliferation cultures were established in MS medium with 20μM BAP Shoots were easily rooted on MS medium with sucrose.

**2.2 *In vitro* shoot multiplication of banana**

Sarker (2005) carried out an experiment on *in vitro* plantlet regeneration of local and exotic banana cultivars. The cultivar Amritasagar gave the highest (2.6/explant) number of shoots and the lowest (1.67/explant) number of shoots was found in Sabri at 30 DAI. BAP at 5.0 mg/l gave the highest number of shoots at all dates.

Ranjan *et al.* (2001) reported that the highest percentage of explants (shoot tip) responded to shoot initiation on MS medium supplemented with 8 mg/l BA, 3 mg/l IAA and 150 mg/l adenine sulphate. The maximum number 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 mg BA/l.

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 inoculated on MS medium supplemented with 6 mg/l BAP, 2 mg/l IAA and 200 mg/l adenine sulphate. They found that shoot initials developed after 3 weeks and were proliferated in reduced concentration (4 mg/l) of BAP.

Khayat *et al.* (1999) carried out an experiment on banana germplasm improvement at Rahan meristem. Some 300 mats of Grand Naine banana *(Musa* spp.) 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 microshoots were produced from the basal lateral sides of the main shoot tip, when cultured on modified MS medium supplemented with 6 mg/l BAP. Induction of roots was achieved on microshoots when cultured on MS medium supplemented with IAA.

Azad and Amin (1999) cultured banana cv. Sabri using shoot tips (1.01.5 cm) of the field grown young suckers on MS medium supplemented with 3.0-5.0 mg/l BAP + 2.0-3.0 mg/l 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 mg/l BAP, 0.5 mg/l IAA and 15% coconut water, those produced shoots with 3 to 5 levels.

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 mg/l BAP, the shoots were then transferred to medium containing 0, 2, 4 or 6 mg/l BAP

for shoot multiplication. The highest number of proliferated shoots was recorded with 6 mg/1 BAP.

Shoot tips from suckers of the dwarf cultivars Basrai and Shrimanti were cultured on MS media supplemented with 5 mg/1 BAP 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, 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 20μM BAP. 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 IAA 4.0mg/l, IBA 10.0 mg/1 and Adenine hemisulphate 200.0 mg/l. 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/1 produced the highest number of shoots in Amritasagar and Mehersagar. In Amritasagar banana (AAA) meristem tip generated the highest number of shoots on MS medium supplemented with 30 μM BAP and it was reported by Khanam *et al.* (1996).



Multiple shoots were induced from shoot tip culture on MS medium supplemented with BAP (2 mg/1) by Ganapathy *et al.* (1995). Shoot tips excised from multiple shoots were subcultured 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 mg/1 BAP for 45 days.

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 mg/I BAP produced more plantlets during culture of banana shoot tips.

Apical meristems were cultured by Gomez and Garcia (1994) on medium supplemented with 0.5 mg/1 cysteine, 30 g/1 sucrose, 8 g/1 agar and 0.5 or 1.0 mg/1 BAP during the initiation stage and with 5.0 or 10.0 mg/1 BAP and/or 0.25 or 0.5 mg/1 BAP at the multiplication stage. They found no differences among the cultures during the initiation stage with or without BAP in the medium. But shoot proliferation was the highest in the medium supplemented with 5.0 mg/1 BAP.

Tulmann *et. al.* (1990) found new buds of banana after shoot tip cultured *in vitro* on medium containing BAP (5 mg/1), sucrose (30 g/1) and agar at pH 5.7. The multiplication rate of banana cv. Poyo micropropagated *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 mg/l BAP. On the other hand, they obtained single shoots when the explants were cultured on MS medium containing 5.0 mg/l BAP.

Cronauer and Krikorian (1985), Dore and Shahijram (1989) observed that aseptic shoot cultures can be established form 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 ruin 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 g/l sucrose, 0.8% bactoagar and 0, 2.5, or 5.0 mg/l BAP, all the floral apices survived. But proliferation of buds was observed only in culture medium containing BAP at 2.5 and 5.0 mg/l for both the varieties.

In case of micropropagation of banana, the effects of different types of explants such as offshoots, rhizomes or aerial organs (leaf, pseudostem or fruit). Kamate and Anno (1987) investigated meristems from same 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

procmbryogenic 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 auxins 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-Tcontaining medium regenerated bipolar embryos.

In other experiments, Ma and Shii (1972), and Dore Swamy *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 Dore Swamy *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 mg/1 thiamine HCl, 0.5 mg/1 nicotinic acid, 0.5 mg/1 Pyridoxine HCl, 25 mg/1 ascorbic acid, 0.7 mg/1 BA and 0.7 mg/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 corms sections and tissue culture derived shoots of Saba banana (*Musa* sp. cv. Saba BBB) formed multiple shoots on MS medium supplemented with 10 mg/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 micropraopagation 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 mg/l BAP.

**2.3 *In vitro* root induction**

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 micro shoots of banana.

Khaldun (2005) reported that the cultivar Amritasagar showed rooting in all concentrations of IAA (1.0, 2.0, 2.5, 3.0 and 4.0 mg/l) used on MS medium. The best (3.67 roots/plantlet) result of root formation was observed at 3.0 mg/l IAA.

Habiba *et al.* (2002) observed that half strength MS supplemented with 2.0 mg/l IBA was the best for root induction in the regenerated shoots.

Vani and Reddy (1999) reported that the shoots were rooted on MS medium containing 2.0 mg/l BAP, 2.0 mg/l 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 mg/l IBA + 0.5-1.0 mg/l IAA + 15% coconut water induced rooting *in vitro* on MS medium with 0.2-0.5 mg/l IBA.

Nandi and Chaudhury (1997) found that the MS medium supplemented with 5 mg IBA/l was the best for root differentiation of the dwarf cultivars Basrai and Shrimanti.

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 mg/l IBA. Whereas, Domingues *et al.* (1995) observed that rooting was stimulated in a nutrient solution supplemented with 0.1 mg/l NAA or IBA.

Raut and Lokhande (1989) found that rooting was induced on MS medium supplemented with 2.0 mg/l IBA and whole plantlets were successfully established in pot. Micro shoots of banana produced sufficient roots in the medium containing half strength MS media supplemented with 2.0 mg/l IBA (Habib, 1994).

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 µM IBA. The plantlets were potted in vermiculite 2-3 weeks after rooting and were successfully transplanted to the fields within 3 months.

Murali and Duncan (1991) observed that basal medium supplemented with 1.0 mg/l IBA induced root in micro shoots of banana. Cronauer and Krikorian (1984b) obtained rooted plantlets by treating with NAA (1.0 mg/l) and activated charcoal (0.02%).

Lameira *et al.* (1990) observed that shoots obtained from explants of variety Prata (AAB) cultured on MS medium with IBA, initiated roots on half strength MS medium with 2.0 mg/l IBA. They also observed that plantlets grew on medium containing vermiculite, organic matter and plant nutrient without growth regulators.

Mateille (1988) reported the shoot elongation and rooting occurred simultaneously on a medium containing 10 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 rooting from meristem occurred from 10 to 12 weeks on BAP and kinetin enriched MS medium.

### 2.4 *Ex vitro* survival of plantlets

Bhuiyan (2007) obtained that *in vitro* rooted shoot of banana cv. Amritasagar survived 85.90% on pot mixture containing sand, sand and cow dung at 1: 1: 1 ratio.

Khaldun (2005) reported that most of the plantlets (77%) regenerated from shoot tip explants of banana cv. Amritasagar and Sabri survived in the field condition.

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). Where 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) reported that when the banana cv Mysore was screened for female fertility. A triploid hybrid was found to contain BSV which confirmed seed transmission of the virus. Therefore, attempts are being made to free Mysore from the virus by meristem culture.

Gomez and Garicia (1994) observed that ten months after transferring to the soil, plantlets obtained from media supplemented with 10 mg/l BAP were larger and had longer petioles than plantlets obtained from media supplemented with 5 mg/l BAP during the multiplication stage.

A comparative study 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 can be used in commercial banana production in Thailand with some advantages.

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%.

# Chapter III

# Materials and Methods

# CHAPTER III

# MATERIALS AND METHODS

The study was carried out at the laboratory of Proshika Tissue Culture Centre Trust (PTCCT), Manikgonj during the period from April, 2008 to November, 2008 to obtain *in vitro* regeneration of banana cv. Amritasagar and Sabri. Materials and Methods followed to conduct the present investigation have been presented in this chapter.

## 3.1 Experimental materials

### 3.1.1 Plant materials

The experimental materials were the shoot tips of the following cultivars of banana.

i) Amritasagar (*Musa sapientum*, Genotype AAA)

ii) Sabri *(Musa sapientum*, Genotype AAA)

The explants were collected from the field of Proshika, Manikgonj. The shoot tip used for the establishment of culture was prepared 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 relatively some major factors. A significant factor is the choice of nutritional components and growth regulators. Both for shoot regeneration and rooting of multiplied shoots, MS medium (Murashige and skoog, 1962) was used with different vitamins and hormonal supplementation. The composition of the 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 4±1$^0$C temperature. The respective media were prepared from the stock solutions.

### 3.2 Preparation of the stock solutions

The first step in the preparation of the medium was the preparation of stock solutions of the various constituents of the medium. As different media constituents were required in different concentrations, separate stock solutions for the macronutrients, micronutrients, irons, vitamins and amino acids, growth regulators etc. were prepared separately for ready use.

#### 3.2.1 Stock solution of macro-nutrients (Soln. A)

Stock solution of macronutrients was prepared with 10 times the final strength of the medium in 1000 ml of distilled water (dw). Ten times the weight of the salts required for one litre of medium were weighed accurately and dissolved thoroughly in 750 ml of distilled water and final volume was made up to one litre by further addition of dw. The stock solution was filtered through a whatman no.1 filter paper to remove all the solid contaminants and solid particles like cellulose dust, cotton etc. The stock solution was poured into a clean plastic container and stored in refrigerator at 4±1$^0$C for ready use.

#### 3.2.2 Stock solution of micro-nutrients (Soln. B)

Stock solution of micronutrients was made up to 100 folds (100x) the final strength of the medium in 1000 ml dw as described earlier for stock solution A. The stock solution was filtered, labeled and stored in a refrigerator at 4±1$^0$C for later use.

#### 3.2.3 Stock solution of iron (Soln. C)

It was made up to 100 folds (100x) the final strength of the medium in 1000ml of dw. Here two constituents, $FeSO_4$ 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 by further addition of dw. Finally, the stock solution

was filtered and stored by wrapping with aluminium foils in a refrigerator at $4\pm1^0$C for later use.

### 3.2.4 Stock solution of vitamins and amino acids (Soln. D)

The following vitamins and amino acids were used for the preparation of MS medium:

Pyridoxine HCl (Vitamins $B_6$)
Thiamine HCl (Vitamins $B_1$)
Myoinositol (Inositol)
Glysine
Nicotinic acid (Vitamins $B_3$)

Each of the above vitamins and amino acids 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 $4\pm1^0$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 $4\pm1^0$C.

### 3.2.5 Stock solution of growth regulators (Soln. E)

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 of tissues and organs. The following growth regulators were used in the present investigation, which were dissolved in appropriate solvent as shown against each of them.

| **Growth regulators (Solute)** | **Solvents** |
|---|---|
| **Auxins** | |
| Indole 3-Butyric Acid (IBA) | 70% ethyl alcohol |
| **Cytokinin** | |
| 6-Benzyl Amino Purine (BAP) | 0.1N NaOH |

To prepare a stock solution of growth regulator, 10 mg of the growth regulator was taken on a clean watch glass then dissolved in 1 litre measuring cylinder and the volume was made up to 100 ml with distilled water. The solution was then poured into a clean glass container and stored at $4\pm1^{0}C$ and used for a maximum period of two months.

### 3.3 Media Preparation

To prepare one litre of the MS medium, the following steps were followed:

I. 100 ml of macronutrients, 10 ml of micronutrients, 10 ml of irons, 10 ml of vitamins and 10 ml 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 into the beaker.

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

IV. Different concentrations of hormonal supplements as required were added to this solution and were mixed thoroughly.

V. Since each hormonal stock solution contained 10 mg of the chemical in 100 ml of solution, to make one litre of medium addition of 10 ml of stocks of any of the hormones, resulted in 1 mg/l concentration of that hormonal supplement. Similarly, for 1.0, 2.0, 3.0, 4.0, 5.0, 6.0 and 7.0 mg/l concentrations of the hormonal supplement 10, 20, 30, 40, 50, 60 and 70 ml of hormonal stock solutions were added respectively to prepare one litre of medium.

VI. The whole mixture was then taken into one liter measuring cylinder to make the exact volume with the further addition of distilled water and poured back to a one litre conical flask and mixed well with the help of a magnetic stirrer.

VII. The 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.

VIII. To solidify the medium 7 gm/l agar was added to the solution. The mixture was then heated in an electric heater cum stirrer with continuous stirring till complete dissolution of agar.

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

### 3.4 Sterilization of culture media

The culture tubes containing media were then autoclaved at 15 psi pressure at $121^0$C for 20 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 aluminum foils were sterilized in a pressure cooker or in an autoclave at a temperature of $121^0$C for 20 minutes at 15 psi pressure.

### 3.6 Sterilizing culture room and transfer area

Initially, the culture room was cleaned by gentle washing all floors and walls with detergent. This was followed by carefully wiped them with 70% ethyl alcohol. The process of sterilization of culture room was repeated at regular intervals. Laminar Airflow Cabinet was usually sterilized by switching on the UV light of the cabinet for 30 minutes and wiping the working surface with 70% ethyl alcohol for 30 minutes before starting the transfer work.

### 3.7 Precautions to ensure aseptic condition

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 scalpels, forceps, 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 cultures.

### 3.8 Culture method

The following culture methods were employed in the present investigation-I. Explant culture 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 of banana cultivars. Small suckers of banana were collected from field and were brought to the preparation room. 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. A number of outer leaves were removed until the shoot measured about 1.50 – 2.00 cm in length and 1.0 cm width at the base.



##### 3.8.1.2 Surface sterilization of explants

The prepared explants were taken into a conical flask and were washed with distilled water containing 1% savlon and 3-4 drops of Tween-80 for 20 minutes to remove dusty substance. This was followed by successive 3 times washing with distilled water to make the materials free from savlon and Tween-80. Subsequently the materials were transferred to running Laminar Airflow Cabinet. The surface sterilization of explants was carried out in 0.1% $HgCl_2$ for different periods (10, 12, 14, 16 minutes) according to nature of explants. Then the materials were washed 3-5 times with distilled water to remove all traces of $HgCl_2$.

### 3.8.1.3 Inoculation of culture

The explants were prepared very carefully in the aseptic condition under laminar airflow cabinet. All the overlapping sheaths and extra rhizome tissue were removed further with a sterile pointed scalpel. The shoot tip explants of about 0.5 cm long with 3-4 leaf primordial were prepared. The individual shoot tip was directly inoculated to each culture vessel and covered with plastic cap. After that the caps were sealed with parafilm.

### 3.8.1.4 Culture conditions

The culture vessels were transferred to growth room and were allowed to grow in controlled environment. The temperature of the growth room was maintained within $25\pm2^{0}$C by air conditioner. A 16-hour light period was maintained by the illumination from white florescent tube light and light intensity was 2000 lux for the growth and development of the cultures.

### 3.8.1.5 Blackening of the explants

Some explants became black in colour within 6-7 days after inoculation. To control further blackening, the blackish tissues on the explants were removed and the shoot tips were transferred to similar fresh medium. It was repeated at 10 days interval for about one month to minimize further blackening of the tissues.

### 3.8.2 Subculture or transfer

### 3.8.2.1 Maintenance of proliferating shoots

Initial subculturing was done when the explants had produced some shoots. For subculturing, the entire samples of *in vitro* shoots were cut into small pieces so that each piece would contain about one shoot. Leaf and blackish or browned 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 shoots

*In vitro* proliferated micro shoots were separated and each of the micro shoots 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 into 2-4 cm in length with 2-3 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 concentrations of hormonal supplements for root induction.

## 3.9 Preparation of pot

Potting mixture containing ground soil, sand and cow dung at the ratio of 1:1:1 was mixed thoroughly and were placed into a pot for growing *in vitro* grown plantlets under *ex vitro* conditions.

## 3.10 Treatments

Two experiments were conducted to assess the effect of different concentrations of BAP and IBA on shoot multiplication and subsequent rooting of the multiplied shoots.

### Experiment 1: Effect of different concentrations of BAP on shoot multiplication of two banana cultivars

In this experiment, the effect of different concentrations of BAP on shoot multiplication of two cultivars of banana was studied. There were 8 levels of BAP (0, 1, 2, 3, 4, 5, 6 and 7 mg/l) were used.

**Experiment 2: Effect of different concentrations of IBA on rooting of meristem derived micro shoots**

In this experiment, the effect of IBA on root formation of the micro shoots was investigated. There were 6 levels of IBA (0, 1, 2, 3, 4 and 5 mg/l) were used.

The experiments were arranged in a Completely Randomized Design (CRD) with 3 replications.

### 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 Relative colour changes of inoculated shoot

#### 3.11.2 Percentage of explants induced shoot

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

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

#### 3.11.3 Number of shoots per explant

Number of shoots per explant was recorded at 10 days interval up to one month of culture and mean number of shoots per explant was calculated by using the following formula,

$$\overline{X} = \frac{\sum x_i}{n}$$

Where,

$\overline{X}$ = Mean of shoots/explant
$\sum$ = Summation
$x_i$ = Number of shoots/ explant
n = Number of observations

### 3.11.4 Length of shoot

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

### 3.11.5 Number of leaves and length of leaves

The number of leaves produced on the plantlet and length of the leaves were counted at 10 days interval up to one month of culture and the mean was calculated using the above mentioned formula.

### 3.11.6 Percentage of shoots developed roots

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

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

### 3.11.7 Number of roots per plantlet

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

### 3.11.8 Length of root

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

### 3.12 Transfer of plantlets from cultured tube to soil

The plantlets with well developed roots were removed from the culture vessels without damaging the roots. The culture medium was washed away from the roots with running tap water. After that, the plantlets were treated with fungicide (1.5-2 g/l Ridomil) for 20 minutes to prevent unwanted fungal or

bacterial growth in the roots. These plantlets were transferred to small polythene bag filled with soil, sand and well decomposed cow dung (1:1:1) and kept in the hardening room for 5-8 days. Then the polythene bags containing plantlets were transferred to net house, where proper care was taken for growth and development of banana plantlets. After 15-20 days, maximum plantlets showed vigorous growth while some were less vigorous. These plantlets were transferred to the field conditions after 30 days where they grew under conditions of normal environment.

### 3.13 Analysis of data

The data for the characters under study were statistically analyzed following Completely Randomized Design (CRD). The analysis of variance was performed and means were compared by Least Significant Difference (LSD) test for interpretation of results.

# Chapter IV

# Results and Discussion

# CHAPTER IV

# RESULTS AND DISCUSSION

Regeneration of banana plantlets through shoot tip culture offers the unique facilities of reproduction protocol with a view to supply planting materials in large quantities especially for large scale cultivation. So the regeneration of banana plantlets through meristem culture and subsequent shoot multiplication could play an important role. In present experiment, *in vitro* regeneration of two banana cultivars through BAP on shoot multiplication and IBA on the subsequent rooting of shoots was studied. Remarkable variations were observed between the cultivars as well as different concentrations of BAP and IBA. The results obtained from this experiment have been presented and discussed under the following headings.

## 4.1 Surface sterilization of plant materials

### 4.1.1 Effect of different strength of $HgCl_2$

After 10 days of sterilization and inoculation, it was observed that when the explants were treated with 0.1% $HgCl_2$ for 14 minutes, 100% shoot tip explants of Amritasagar and Sabri were found free from any contamination (Table 1). On the other hand, the percentage of contaminated explants increased at 20 days after sterilization and inoculation because there were endogenous bacteria in the meristem of explants that expressed later. When $HgCl_2$ was used for short durations (10 and 12 minutes), the treatments failed to kill the microorganisms attached to the surface of field grown explants. Among the periods of treatment, the highest percent of explants survived at 14 minutes (Table 1). Treatments for more than 14 minutes duration survival percentage was decreased and tissue became damage. So, concentrations of disinfectants and soaking time are adjusted according to the resistance of the explants to sterilants.

**Table 1: Standardization of $HgCl_2$ treatment period for surface sterilization of shoot tips of Amritsagar and Sabri**

| Cultivars | Treatment period of 0.1% $HgCl_2$ (min.) | No. of explants cultured | % of explants contaminated after | | % of explants survived after |
|---|---|---|---|---|---|
| | | | 10 days | 20 days | 20 days |
| Amritasagar | 10 | 10 | 60 | 80 | 20 |
| Sabri | 10 | 10 | 50 | 70 | 30 |
| Amritasagar | 12 | 10 | 30 | 40 | 60 |
| Sabri | 12 | 10 | 30 | 40 | 60 |
| Amritasagar | 14 | 10 | - | 10 | 90 |
| Sabri | 14 | 10 | - | 10 | 90 |
| Amritasagar | 16 | 10 | - | 20 | 80 |
| Sabri | 16 | 10 | - | 30 | 70 |

In case of *in vitro* grown explants, they were excised and inoculated directly in the media because they were in aseptic condition. But in case of field grown explants (Plate 1&2) surface sterilization was made before the inoculation due to the presence of loose contaminants on the explants surface. In our environment, microbes like bacteria, fungi and virus are the most common contaminants observed in cultures. To overcome this problem scientist used different types of sterilizing agents with different concentrations. The duration of sterilization is also different with different workers. Vessey and Revera (1981) used 0.25% sodium hypochlorite. Dore *et al.* (1983) used chlorine saturated water for 15-20 minutes. Sodium hypochlorite (0.05%) was also used by Cronauer and Krikorian (1984a).

In present experiment, explants were surface sterilized with 0.1% $HgCl_2$. Contamination free culture with higher surviving ability was achieved by treating the explants with 0.1% $HgCl_2$ for 14 minutes. It indicated that the duration of soaking of explants and the concentrations of the disinfectants were important factors to sterilize the explants.

**Plate 1: Plant material (sucker) of Amritasagar**

**Plate 2: Plant material (sucker) of Sabri**

### 4.2 Establishment of explants in the culture media

#### 4.2.1 Relative colour changes of explants

After inoculation to the culture media, the shoot tips showed creamy white appearance at first sight and gradually became brown to light green, green and finally dark green on MS medium supplemented with different concentrations of BAP (Plate 3 & 4). The extents of colour changes were recorded at 10 days after inoculation (DAI) and 20 DAI. The response of colour change of the explants of Amritasagar and Sabri varied in respect of time and concentrations of BAP used. The explants of Amritasagar became dark green at 3.0 mg/l and 5.0 mg/l at 10 DAI but at 1.0 mg/l, 2 mg/l, 4 mg/l, 6.0 mg/l and 7.0 mg/l BAP the inoculated explants became green and at control it turned light green in colour. All the explants treated with BAP turned dark green in colour except in control i.e., 0.0 mg/l BAP at 20 DAI (Table 2).

In case of Sabri, relative colour changes were different from the Amritasagar and the rate of colour change was slow (Table 2). After 10 days the colour was green for BAP concentrations of 3.0 mg/l, 6 mg/l and 7.0 mg/l while for 1.0 mg/l, 2.0 mg/l, 4 mg/l and 5.0 mg/l BAP the explants turned light green but in control the explants appeared brown in colour. On the other hand, after 20 days of inoculation all the explants turned dark green in colour for all the concentrations of BAP used except in control and 1.0 mg/l BAP. Rabbani *et al.* (1996) also found the same result. In control, the colour was light green in Sabri and green in Amritasagar after 20 DAI. Therefore, it was clear that BAP was essential for early greening of the banana cultivars.

The above results appeared that the colour changes of inoculated explants showed clear variation in the same treatment for different varieties. It was probably due to genotype variation. Ranjan *et al.* (2001) reported similar results while working with 10 cultivars of bananas. He also reported that the presence of cytokinin in the medium was essential for greening of banana shoot tip explants.

**Table 2: Effect of different concentrations of BAP on relative colour changes of banana cv. Amritasagar and Sabri**

| Treatments | Banana cultivars | | | |
|---|---|---|---|---|
| | Amritasagar | | Sabri | |
| Conc. of BAP (mg/l) | 10 days | 20 days | 10 days | 20 days |
| 0.0 | + | ++ | B | + |
| 1.0 | ++ | +++ | + | ++ |
| 2.0 | ++ | +++ | + | +++ |
| 3.0 | +++ | +++ | ++ | +++ |
| 4.0 | ++ | +++ | + | +++ |
| 5.0 | +++ | +++ | + | +++ |
| 6.0 | ++ | +++ | ++ | +++ |
| 7.0 | ++ | +++ | ++ | +++ |

**Legend:** B Indicates Brown

+ Indicates Light Green

++ Indicates Green

+++ Indicates Dark Green

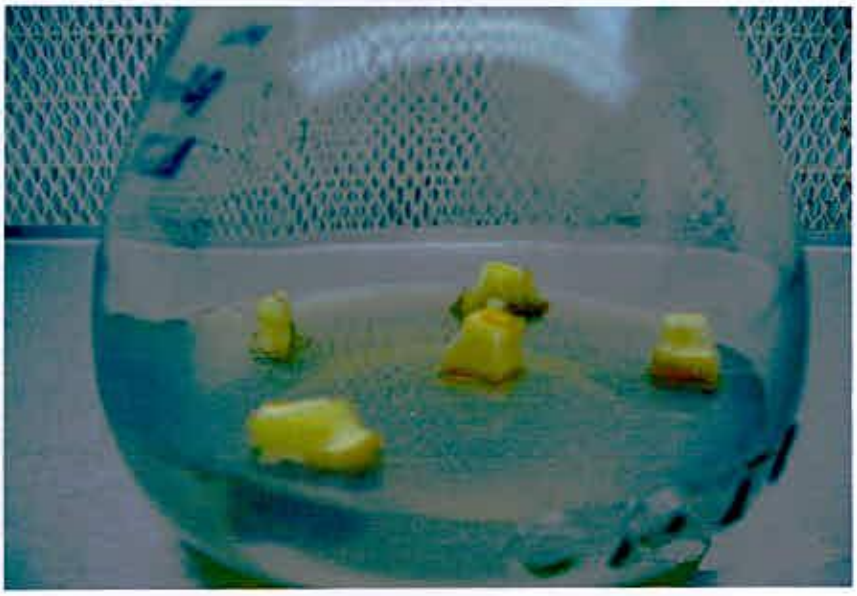


**Plate 3: A just inoculated shoot tip isolated from a sucker showed creamy white colour**

**Plate 4: Inoculated shoot tip became dark green in colour**

### 4.2.2 Survivability

The meristem cultured on MS medium supplemented with different concentrations of BAP had significant effect on percentage of survivability. The highest percentage of survivability was recorded when the explant was cultured on MS medium containing 5 mg/l BAP and the lowest percentage of survivability was found in MS medium without hormone for both varieties. Amritasagar and Sabri showed 90% and 80% survivability respectively at 5 mg/l BAP (Table 3). However, Amritasagar showed better survivability over Sabri for all the treatments.

### 4.2.3 Regeneration of single shoot from meristem explants

It is highly expected to get multiple shoot through tissue culture practices but multiple shoot formation is impossible without forming a single shoot. So, single shoot regeneration is a preliminary event of *in vitro* regeneration practices.

It was observed that the Amritasagar developed single shoot in all the treatments except the control. The highest percentage (50%) of explants developed single shoot in Amritasagar at 5.0 mg/l BAP whereas the lowest percentage (20%) was at 1.0 mg/l BAP. There was variation in days required for single shoot regeneration at different concentrations of BAP used.

In case of Sabri, the highest percentage (30%) of explants produced single shoot at 5.0 mg/l BAP whereas the lowest percentage (10%) was at 1.0 mg/l BAP. It was observed from the results that single shoot regeneration was lower in case of Sabri compared to Amritasagar and the average time required for single shoot regeneration was higher than Amritasagar (Table 3). Rabbani *et al.* (1996) found the same result at all concentrations of BAP in case of time requirement for single shoot formation.

Table 3: Response of *in vitro* cultured meristem cv. Amritasagar and Sabri at different concentrations of BAP. Data were recorded at 20 DAI

| Conc. of BAP (mg/l) | Banana cultivars | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Amritasagar | | | | Sabri | | | |
| | Tuber like structure(%) | Survivability (%) | Single shoot from meristem (%) | Days to single shoot development | Tuber like structure (%) | Survivability (%) | Single shoot from meristem (%) | Days to single shoot development |
| 0.0 | 20 | 50 | - | - | 10 | 40 | - | - |
| 1.0 | 30 | 70 | 20 | 17 | 30 | 50 | 10 | 20 |
| 2.0 | 30 | 70 | 30 | 16 | 20 | 70 | 20 | 19 |
| 3.0 | 40 | 80 | 40 | 15 | 30 | 70 | 20 | 17 |
| 4.0 | 50 | 80 | 40 | 13 | 30 | 60 | 30 | 15 |
| 5.0 | 60 | 90 | 50 | 12 | 40 | 80 | 30 | 14 |
| 6.0 | 40 | 70 | 40 | 14 | 30 | 70 | 20 | 15 |
| 7.0 | 30 | 60 | 30 | 15 | 20 | 50 | 20 | 15 |

*Ten explants were inoculated/treatment

The results indicated that the percentage of single shoot regeneration increased with the increase of BAP concentration up to 5.0 mg/l and there after declined. The cultivars responded to different concentrations of BAP were different (Plate 5 & 6). Response of Amritasagar was the highest (50%) followed by Sabri (30%) at 5.0 mg/l BAP. Rehana (1999) found that time requirement for single shoot formation to be 10-15 days, which was close to the present investigation.

### 4.2.4 Tuber like structure developed from mersitem explants

The cultured meristem formed hard meristematic tuber like structure in regeneration media containing different concentrations of BAP. It was found that the hard meristematic tuber formation was the highest in explants placed on MS medium supplemented with 5.0mg/l BAP and the lowest without any hormone for both the cultivars. In Amritasagar and Sabri, the highest rate of forming tuber like structure were 60% and 40% respectively on MS medium with 5.0 mg/l BAP. The lowest percentage of forming tuber like structure was found, i.e. 20% and 10% in Amritasagar and Sabri respectively on MS medium without any hormone. Therefore, Amritasagar showed better performance in formation of tuber like structure over Sabri. Similar results were also obtained by Habib (1994), who observed that some tuber like structures formed from the base of the shoot during shoot multiplication. These structures of banana are very good for germplasm preservation and can be utilized for further multiplication and subsequently mass proliferation of ideal plantlets for commercial exploitation.

**Plate 5: Single shoot of Amritasagar produced on MS medium containing 5.0 mg/l BAP at 30 days after inoculation**

**Plate 6: Single shoot of Sabri produced on MS medium containing 5.0 mg/l BAP at 30 days after inoculation**

### 4.3 Effect of different concentrations of BAP on shoot proliferation from shoot tip explants

To achieve the ultimate goal of plant regeneration and subsequent shoot proliferation from shoot tip derived plantlets of banana cultivars, MS medium supplemented with different concentrations of BAP was used. The explants developed single shoot within 30 days of inoculation on the culture medium supplemented with different concentrations of BAP were decapitated to inhibit the apical dominance and to produce multiple shoots (Plate 7, 8, 9 & 10). Data were recorded at 10, 20 and 30 DAI and the results obtained from this experiment have been discussed under the following headings.

#### 4.3.1 Number of shoots per explant

Different cultivars and concentrations of BAP significantly influenced the number of shoots produced per explant. Data were recorded at 10, 20 and 30 DAI. The response of cultivars on shoot proliferation was found significant. The results showed (Figure 1) that the cultivar Amritasagar produced 0.38 shoots per explant at 10 DAI whereas, no response was observed in Sabri. On the other hand, Amritasagar gave the highest (1.58/explant) number of shoots while the lowest (1.00/explant) number of shoots was found in Sabri at 30 DAI (Figure 1). The number of shoots produced per explant increased with the increase of DAI but the rate of increase was higher in Amritasagar than Sabri. These results indicated that the Amritasagar was superior to Sabri for this parameter.

The combined effect of cultivars and different concentrations of BAP on shoot proliferation has been presented in Table 4. The results showed that both cultivars gave the highest number of shoots at 5.0 mg/l BAP among the concentrations of BAP used in MS medium at different DAI. The number of shoots increased with the increase of BAP concentration up to 5.0 mg/l and then decreased. No response took place at control.

**Table 4: Combined effect of cultivars and concentrations of BAP on number of shoots**

| Cultivars | Conc. of BAP (mg/l) | Number of shoots per explant at different days after inoculation | | |
|---|---|---|---|---|
| | | 10 days | 20 days | 30 days |
| Amritasagar | 0 | 0.00 | 0.00 | 0.00 |
| | 1 | 0.00 | 0.33 | 0.60 |
| | 2 | 0.30 | 0.62 | 1.65 |
| | 3 | 0.65 | 1.33 | 2.25 |
| | 4 | 0.67 | 1.33 | 2.33 |
| | 5 | 1.00 | 1.72 | 3.50 |
| | 6 | 0.36 | 0.71 | 1.33 |
| | 7 | 0.30 | 0.37 | 1.00 |
| Sabri | 0 | 0.00 | 0.00 | 0.00 |
| | 1 | 0.00 | 0.00 | 0.33 |
| | 2 | 0.00 | 0.32 | 1.30 |
| | 3 | 0.00 | 0.65 | 1.32 |
| | 4 | 0.00 | 0.68 | 1.35 |
| | 5 | 0.00 | 1.00 | 2.00 |
| | 6 | 0.00 | 0.69 | 1.00 |
| | 7 | 0.00 | 0.67 | 0.68 |
| LSD (0.05) | | 0.479 | 0.507 | 0.611 |

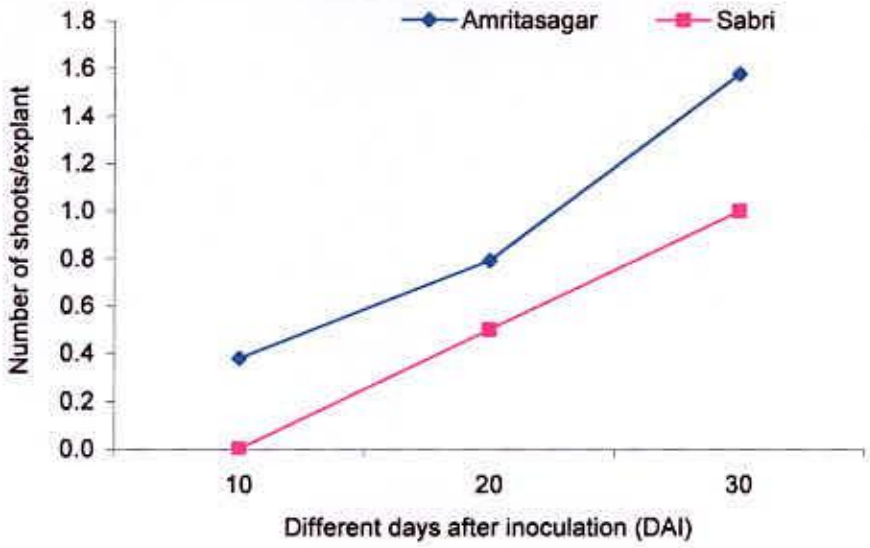



**Figure 1: Response of cultivars on number of shoots at different days after inoculation**

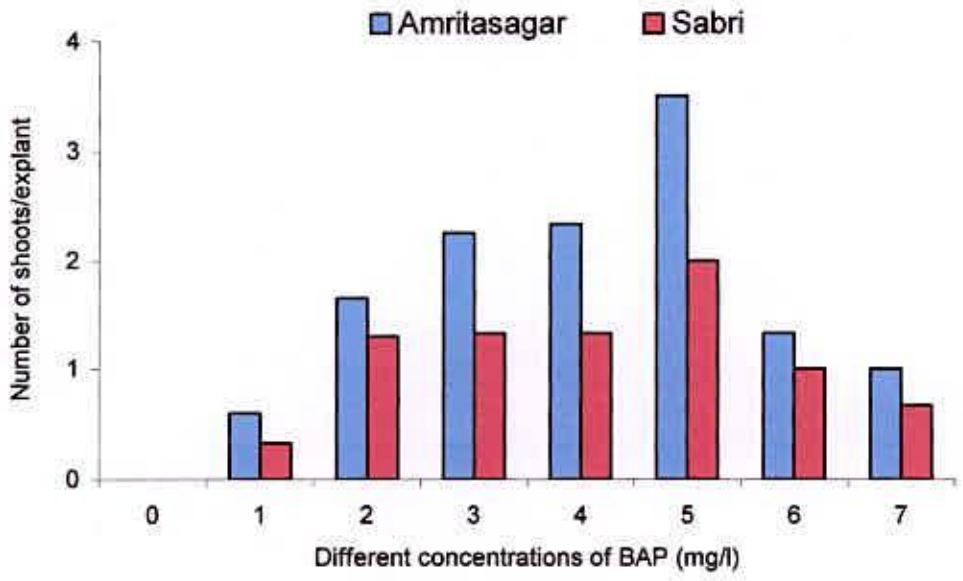



**Figure 2: Combined effect of cultivars and different concentrations of BAP on number of shoots at 30 days after inoculation**

**Plate 7: Multiple shoots of Amritasagar produced on MS medium containing 5.0 mg/l BAP at 10 days after inoculation**

**Plate 8: Multiple shoots of Sabri produced on MS medium cointaining 5.0 mg/l BAP at 10 days after inoculation**

**Plate 9: Multiple shoot of Amritasagar produced on MS medium containing 5.0 mg/l BAP at 30 days after inoculation**

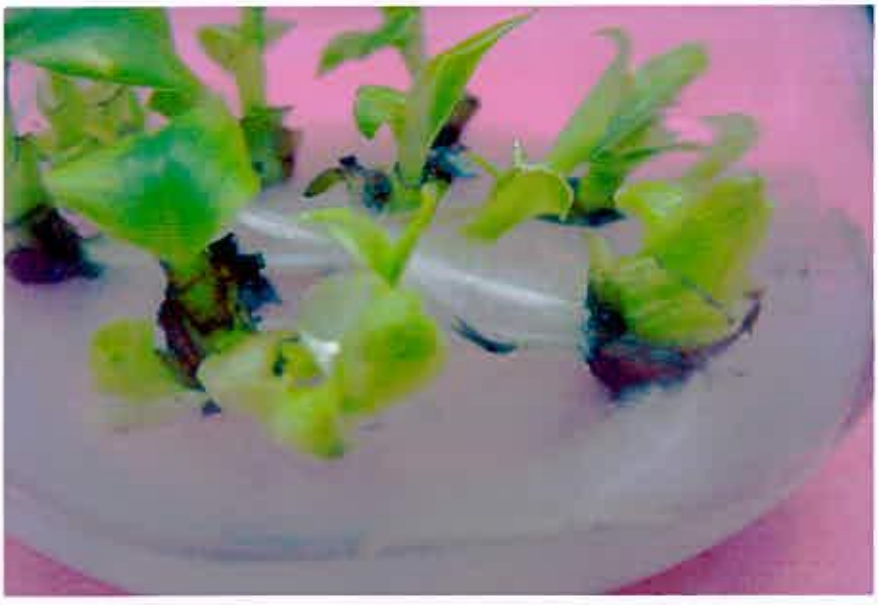


**Plate 10: Multiple shoot of Sabri produced on MS medium containing 5.0 mg/l BAP at 30 days after inoculation**

Among the concentrations of BAP, 5.0 mg/l showed the highest shoot proliferation (3.50/explant) of Amritasagar followed by 2 shoots/explant of Sabri at 30 DAI (Figure 2).

From the above results, it is clear that 5.0 mg/l BAP showed the best performance and Amritasagar was better variety than Sabri. The results of the present investigation agreed with the finding of Rabbani *et al.* (1996) who reported that BAP at the rate of 5.0 mg/l produced the highest number of shoots in Amritasagar and Mehersagar. In another study, banana (AAA) meristem tip generated the highest number of shoots on MS medium supplemented with 6 mg/l BAP in the cultivar Amritasagar (Khanam *et al.*, 1996). Domingues *et al.* (1995) was found the same results with 5.0 mg/l BAP concentration.

### 4.3.2 Shoot length

Shoot length also influenced by cultivars and different concentrations of BAP. Data were recorded at 10, 20 and 30 DAI and results have been presented in Table 5. The response of cultivars showed that Amritasagar produced longer shoot (1.68 cm) compared to Sabri (1.24 cm) at 30 DAI (Figure 3). Besides, Amritasagar produced longer shoot than Sabri at 10 and 20 DAI. These results indicated that Amritasagar performed better than Sabri for the increment of shoot.

The combined effect of cultivars and different BAP concentrations showed that the length of shoot varied due to varieties and concentrations. Among the concentrations of BAP, 5.0 mg/l produced the longest shoot in both cultivars but the cultivar Amritasagar produced longer shoot compared to Sabri at 10, 20 and 30 DAI. The cultivar Amritasagar produced shoot 2.64 cm in length at 5.0 mg/l BAP at 30 DAI, whereas the cultivar Sabri produced shoot 2.16 cm in length at the same concentration of BAP at 30 DAI (Figure 4).

These results indicated that 5.0 mg/l BAP was the best among all concentrations of BAP and Amritasagar showed better performance over Sabri for this parameter. Rabbani *et al.* (1996) found similar results from 5.0 mg/l BAP supplemented MS medium. Khanam *et al.* (1996) obtained the longest shoot in banana on MS medium supplemented with 5.0 mg/l BAP treatment.

**Table 5: Combined effect of cultivars and concentrations of BAP on length of shoots**

| Cultivars | Conc. of BAP (mg/l) | Shoot length (cm) at different days after inoculation | | |
|---|---|---|---|---|
| | | 10 days | 20 days | 30 days |
| Amritasagar | 0 | 0.00 | 0.00 | 0.00 |
| | 1 | 0.00 | 0.09 | 0.86 |
| | 2 | 0.04 | 0.71 | 1.82 |
| | 3 | 0.08 | 1.30 | 2.35 |
| | 4 | 0.09 | 1.35 | 2.47 |
| | 5 | 0.14 | 1.36 | 2.64 |
| | 6 | 0.06 | 0.91 | 2.13 |
| | 7 | 0.00 | 0.42 | 0.96 |
| Sabri | 0 | 0.00 | 0.00 | 0.00 |
| | 1 | 0.00 | 0.00 | 0.33 |
| | 2 | 0.00 | 0.30 | 1.15 |
| | 3 | 0.00 | 0.70 | 1.60 |
| | 4 | 0.00 | 0.73 | 1.82 |
| | 5 | 0.00 | 0.90 | 2.16 |
| | 6 | 0.00 | 0.26 | 1.54 |
| | 7 | 0.00 | 0.23 | 0.95 |
| LSD (0.05) | | 0.074 | 0.341 | 0.544 |

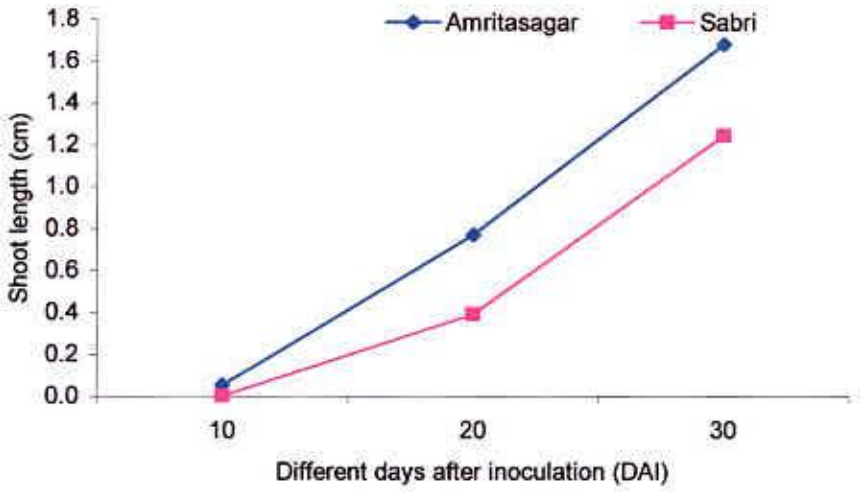



**Figure 3: Response of cultivars on shoot length at different days after inoculation**

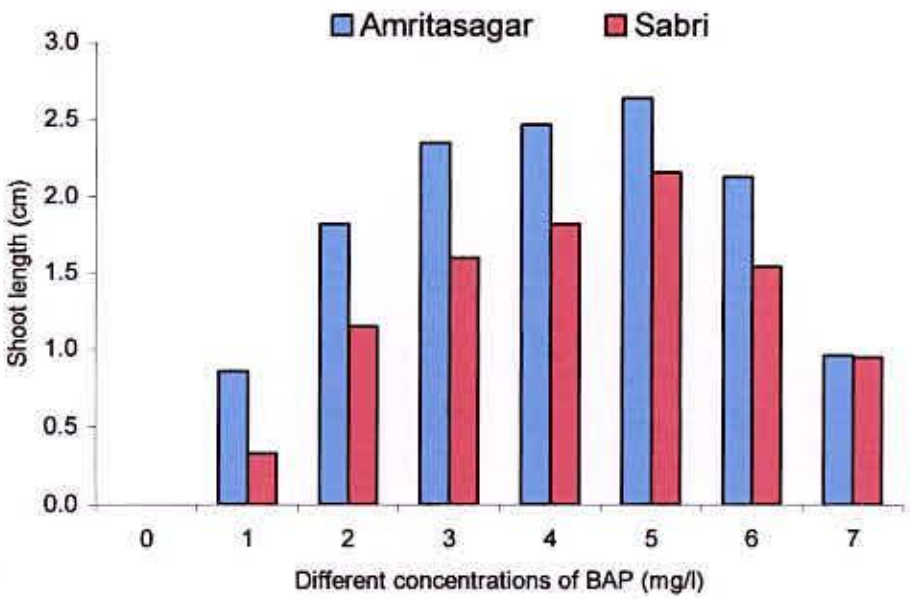



**Figure 4: Combined effect of cultivars and different concentrations of BAP on shoot length at 30 days after inoculation**

### 4.3.3 Number of leaves per plantlet

Number of leaves per plantlet varied significantly on MS medium supplemented with different concentrations of BAP used. The cultivar Amritasagar produced 0.58 leaves per plantlet at 10 DAI but no response took place in case of Sabri. Besides, Amritasagar responded more in the formation of leaves (2.35/plantlet) compared to Sabri (1.48/ plantlet) at 30 DAI (Figure 5). These results indicated that Amritasagar was superior to Sabri in respect to production of leaves per plantlet.

The combined effect of cultivars and different concentrations of BAP on leaf formation has been presented in Table 6. Among the concentrations of BAP, 5.0 mg/l showed the highest number of leaves in both the cultivars at different DAI. The cultivar Amritasagar produced 4.11 leaves per plantlet at 5.0 mg/l BAP at 30 DAI, while the Sabri produced 2.67 leaves per plantlet at the same level of BAP and DAI (Figure 6). From the results (Table 6) it was found that leaf number per plantlet increased with the increase of BAP concentration to 5.0 mg/l and then decreased in both the cultivars. These results indicated that 5.0 mg/l was the best treatment for increasing number of leaves per plantlet. The results of the present experiment agreed with the findings of Rabbani *et al.* (1996) who obtained the same results from 5.0 mg/l BAP, which are close to these findings.

**Table 6: Combined effect of cultivars and different concentrations of BAP on number of leaves**

| Cultivars | Conc. of BAP(mg/l) | Number of leaves per plantlet at different days after inoculation | | |
|---|---|---|---|---|
| | | 10 days | 20 days | 30 days |
| Amritasagar | 0 | 0.00 | 0.00 | 0.00 |
| | 1 | 0.00 | 0.67 | 1.17 |
| | 2 | 0.50 | 1.17 | 2.33 |
| | 3 | 1.00 | 2.17 | 3.50 |
| | 4 | 1.00 | 2.33 | 3.50 |
| | 5 | 1.50 | 2.50 | 4.11 |
| | 6 | 0.67 | 1.00 | 2.17 |
| | 7 | 0.00 | 0.50 | 1.50 |
| Sabri | 0 | 0.00 | 0.00 | 0.00 |
| | 1 | 0.00 | 0.00 | 0.50 |
| | 2 | 0.00 | 0.50 | 2.00 |
| | 3 | 0.00 | 1.00 | 2.00 |
| | 4 | 0.00 | 1.19 | 2.17 |
| | 5 | 0.00 | 1.67 | 2.67 |
| | 6 | 0.00 | 1.17 | 1.50 |
| | 7 | 0.00 | 1.15 | 1.00 |
| LSD (0.05) | | 0.316 | 0.864 | 0.863 |

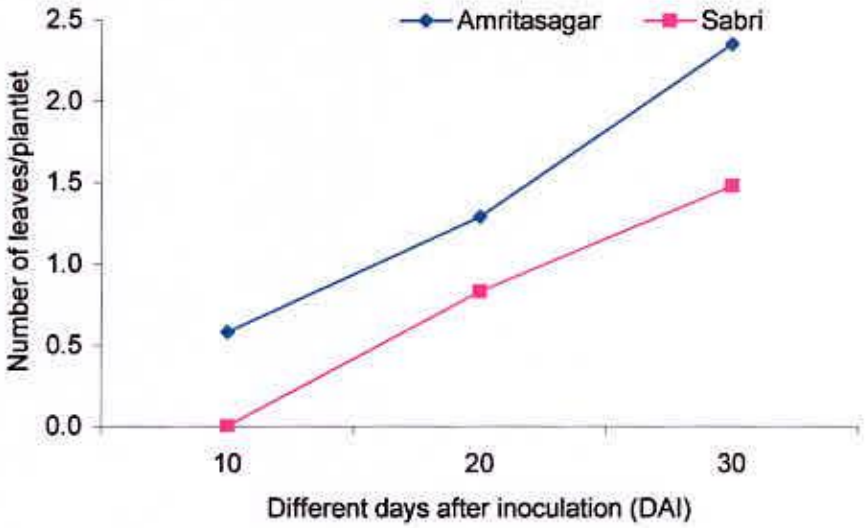



**Figure 5: Response of cultivars on number of leaves at different days after inoculation**

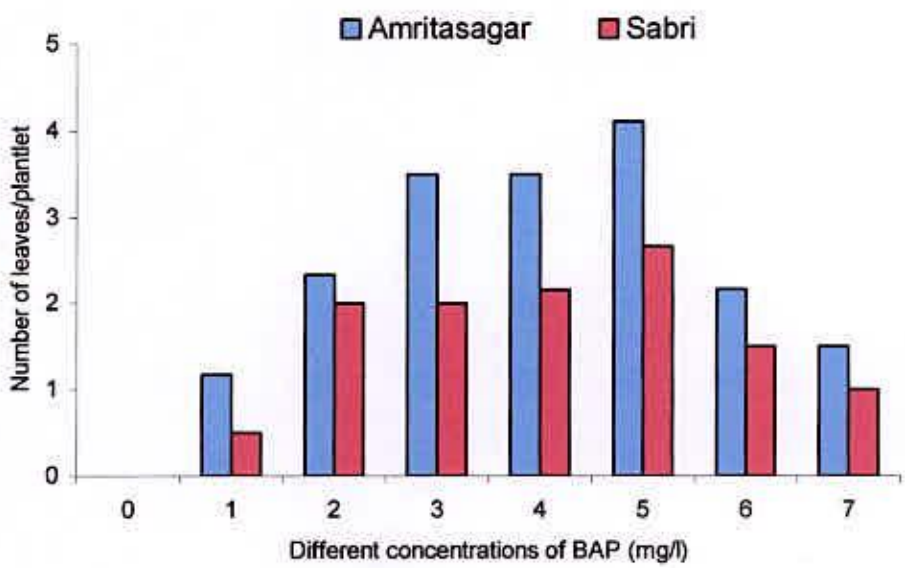



**Figure 6: Combined effect of cultivars and different concentrations of BAP on number of leaves at 30 days after inoculation**

### 4.3.4 Leaf length

The length of leaves produced per plantlet varied on MS medium supplemented with different cultivars and concentrations of BAP used. The response of cultivars on leaf increment at different DAI was found significant. The results presented in Figure 7 showed that Amritasagar produced longer leaf (1.39 cm) compare to Sabri (1.08 cm) at 30 DAI. Besides, Amritasagar produced 0.04 cm leaf in length at 10 DAI but the cultivar Sabri gave no response at that DAI. Moreover, leaf length increased with the increase of DAI in both the cultivars. These results indicated that Amritasagar was superior to Sabri in respect of leaf length per plantlet.

The combined effect of cultivars and different concentrations of BAP on leaf length have been presented in Table 7. Among the concentrations of BAP, 5.0 mg/l showed the longest leaf and 1.0 mg/l showed the shortest leaf in both the cultivars at different DAI. The cultivar Amritasagar produced 2.29cm leaf at 5.0 mg/l BAP at 30 DAI, while the Sabri produced 1.86 cm leaf in length (Figure 8). The results indicated that there was a sharp increase in leaf length at different DAI. Moreover, leaf length increased with the increase of BAP concentration up to 5.0 mg/l and there after declined. These results indicated that 5.0 mg/l was the best treatment for this parameter. These results were in partial support of Rabbani *et al.* (1996) who obtained the longest leaf with 5.0 mg/l each of BAP and Kinetin. The variation of length might be due to the differences of species and inoculated explant. Khanam *et al.* (1996) reported the same results.

**Table 7: Combined effect of cultivars and concentrations of BAP on leaf length**

| Cultivars | Conc. of BAP (mg/l) | Leaf length (cm) at different days after inoculation | | |
|---|---|---|---|---|
| | | 10 days | 20 days | 30 days |
| Amritasagar | 0 | 0.00 | 0.00 | 0.00 |
| | 1 | 0.00 | 0.04 | 0.72 |
| | 2 | 0.03 | 0.55 | 1.48 |
| | 3 | 0.07 | 1.05 | 1.85 |
| | 4 | 0.07 | 1.11 | 1.91 |
| | 5 | 0.11 | 1.21 | 2.29 |
| | 6 | 0.05 | 0.60 | 1.72 |
| | 7 | 0.00 | 0.12 | 0.78 |
| Sabri | 0 | 0.00 | 0.00 | 0.00 |
| | 1 | 0.00 | 0.00 | 0.53 |
| | 2 | 0.00 | 0.14 | 0.93 |
| | 3 | 0.00 | 0.41 | 1.29 |
| | 4 | 0.00 | 0.56 | 1.48 |
| | 5 | 0.00 | 0.72 | 1.86 |
| | 6 | 0.00 | 0.19 | 1.46 |
| | 7 | 0.00 | 0.13 | 0.71 |
| LSD (0.05) | | 0.053 | 0.345 | 0.380 |

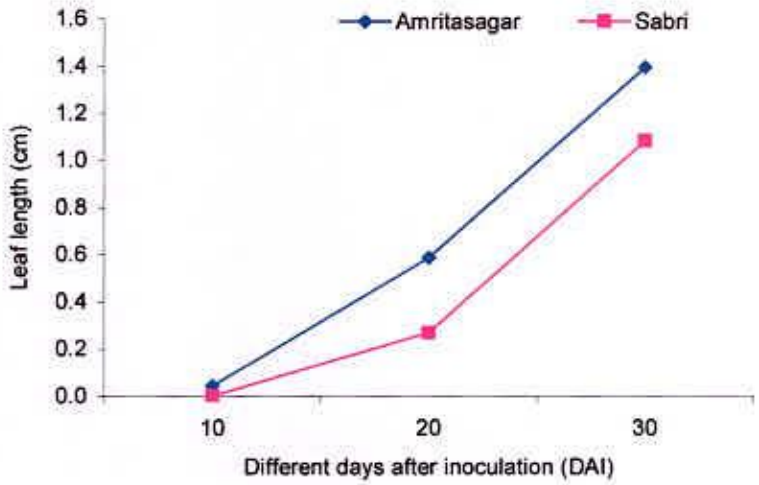



**Figure 7: Response of cultivars on leaf length at different days after inoculation**

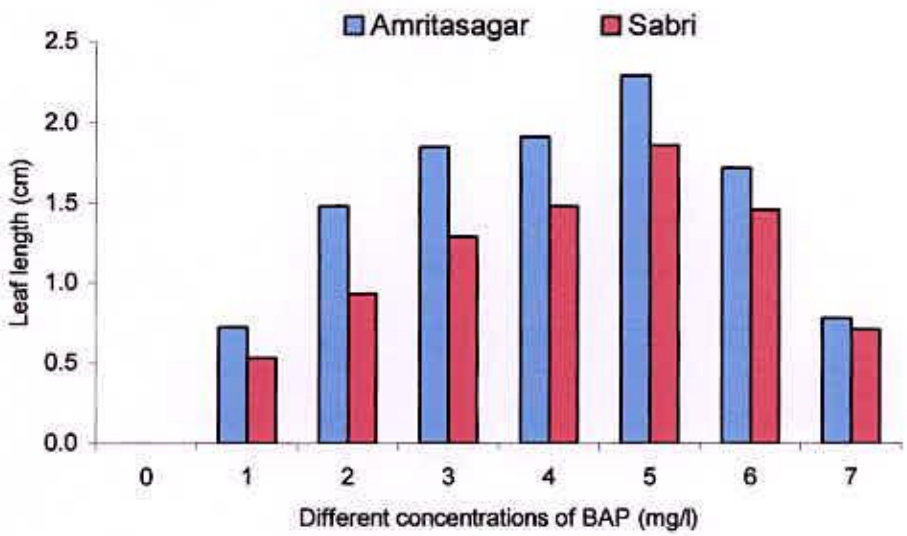



**Figure 8: Combined effect of cultivars and different concentrations of BAP on leaf length at 30 days after inoculation**

### 4.4 Effect of IBA on the induction of roots from multiplied shoots of banana cv. Amritasagar and Sabri

The regenerated shoots were sub cultured on MS medium supplemented with different concentrations of IBA in order to allow root formation. The number of roots and length of roots varied with cultivars and different concentrations of IBA used (Plate 11, 12& 13). The results obtained from this experiment have been discussed under the following headings.

#### 4.4.1 Number of roots per plantlet

The number of roots produced per plantlet varied on MS medium supplemented with different concentrations of IBA used. The response of cultivars on number of roots per plantlet at different DAI was found significant. The cultivar Amritasagar produced more number of roots compared to Sabri at different DAI. The Amritasagar produced 2.78 roots per plantlet at 30 DAI, while the cultivar Sabri produced 1.8 roots per plantlet at the same DAI (Figure 9). Besides, the number of roots per plantlet increased with the increase of DAI. These results indicated that the cultivar Amritasagar showed better performance in root formation over Sabri.

The combined effect of cultivars and different concentrations of IBA on root formation at different DAI was found significant. The results (Table 8) showed that among the concentrations of IBA, 3.0 mg/l gave the highest number of roots per plantlet and 1.0 mg/l gave the lowest number of roots per plantlet in both the cultivars at different DAI whereas no response was observed in control. The number of roots produced per plantlet increased with the increase of IBA concentration up to 3.0 mg/l and there after decreased. The cultivar Amritasagar produced more number of roots per plantlet (4.10/plantlet) than Sabri (3.63/plantlet) at 3.0 mg/l IBA at 30 DAI (Figure 10). These results indicated that 3.0 mg/l IBA was the best treatment among all the concentrations of BAP for this parameter. Habib (1994) and Rabbani *et al.* (1996) obtained the similar result. Raut and Lokhande (1989) also found the same result.

**Table 8: Combined effect of cultivars and concentrations of IBA on root number and root length**

| Cultivars | Conc. of IBA (mg/l) | Roots per plantlet at different days after inoculation | | | Root length (cm) at different days after inoculation | | |
|---|---|---|---|---|---|---|---|
| | | 10 days | 20 days | 30 days | 10 days | 20 days | 30 days |
| Amritasagar | 0 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 1 | 0.42 | 1.00 | 1.27 | 0.10 | 0.57 | 1.14 |
| | 2 | 1.60 | 2.92 | 3.21 | 0.45 | 1.20 | 2.30 |
| | 3 | 1.67 | 3.83 | 4.10 | 0.98 | 2.20 | 3.60 |
| | 4 | 1.74 | 2.60 | 3.72 | 0.90 | 1.55 | 2.72 |
| | 5 | 0.83 | 2.08 | 3.01 | 0.85 | 1.60 | 2.00 |
| Sabri | 0 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| | 1 | 0.00 | 0.47 | 0.60 | 0.00 | 0.40 | 0.90 |
| | 2 | 0.42 | 1.25 | 2.42 | 0.25 | 0.76 | 2.07 |
| | 3 | 1.08 | 2.50 | 3.63 | 0.58 | 1.90 | 3.10 |
| | 4 | 0.93 | 1.75 | 2.42 | 0.47 | 1.20 | 1.72 |
| | 5 | 0.83 | 1.33 | 2.30 | 0.46 | 1.02 | 1.35 |
| LSD (0.05) | | 0.431 | 0.692 | 0.939 | 0.223 | 0.357 | 0.288 |

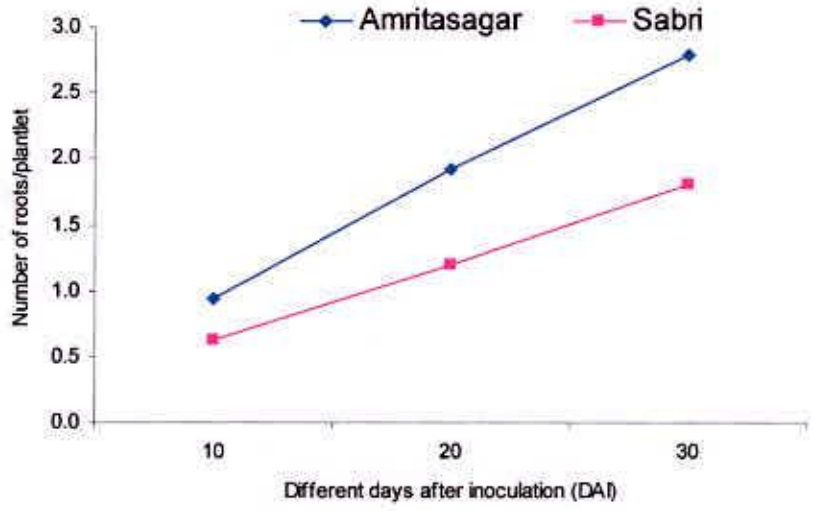



**Figure 9: Response of cultivars on number of roots at different days after inoculation**

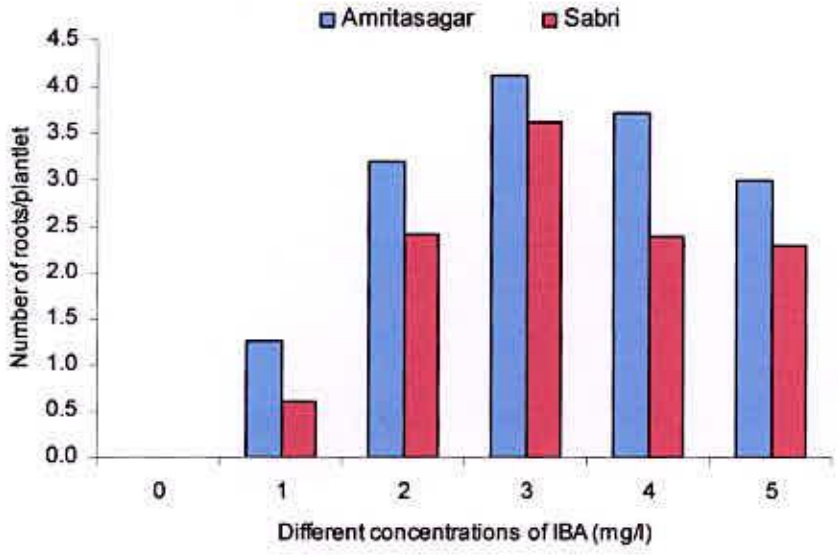



**Figure 10: Combined effect of cultivars and different concentrations of IBA on number of roots at 30 days after inoculation**

**Plate 11: *In vitro* micro shoots for root induction**

**Plate 12: *In vitro* root induction of Amritasagar at 3.0 mg/l IBA after 30 days of culture**

**Plate 13: *In vitro* root induction of Sabri at 3.0 mg/l IBA after 30 days of culture**

### 4.4.2 Root length

The response of cultivars on the increase of root in length at different DAI was found significant. There was a sharp increasing trend in root length at different DAI in both the cultivars but the increment of root length of Amritasagar was superior to Sabri at 10, 20 and 30 DAI (Figure 11). At 30 DAI, the Amritasagar produced roots 2.90 cm in length while the Sabri produced 2.10 cm roots. These results indicated that the cultivar Amritasagar was better than Sabri on the increment of root length.

A significant variation was found among the concentrations of IBA used on MS medium. Among the concentrations of BAP, 3.0 mg/l produced the longest root and 1.0 mg/l produced the shortest root in both the cultivars at different DAI whereas no response was observed in control. The length of roots increased with the increase of IBA concentration up to 3.0 mg/l and there after decreased. The cultivar Amritasagar produced longer roots (3.60 cm) than Sabri (3.10 cm) at 3.0 mg/l IBA at 30 DAI (Figure 12). So, the present results revealed that 3.0 mg/l IBA was the best treatment among all the concentrations for this parameter. The present results agreed with the findings of Bhaskar *et al.* (1993).

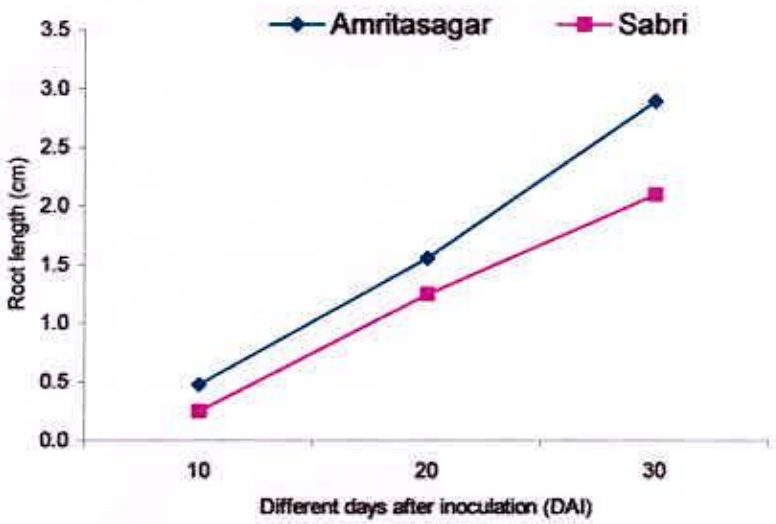



**Figure 11: Response of cultivars on root length at different days after inoculation**

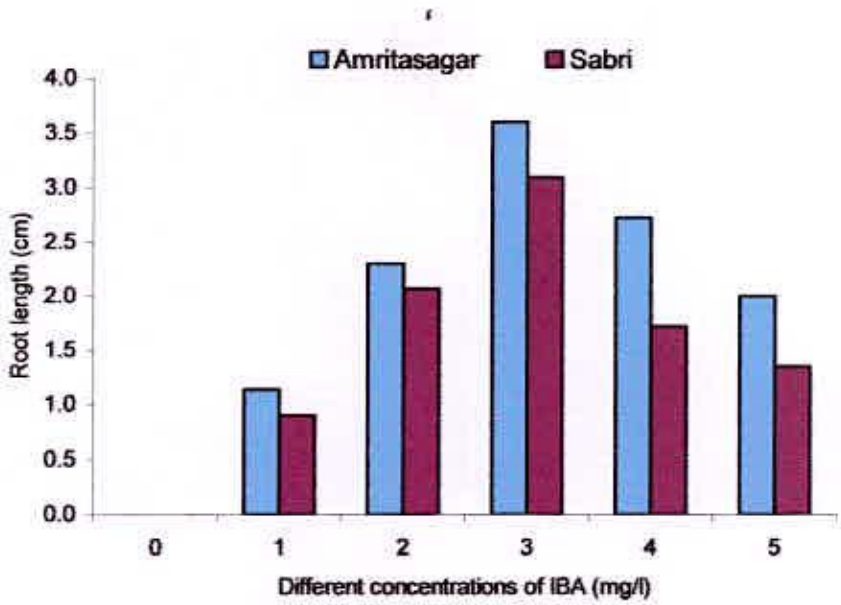



**Figure 12: Combined effect of cultivars and different concentrations of IBA on root length at 30 days after inoculation**

### 4.5 *Ex vitro* hardening of plantlets

The plantlets with well developed roots were removed from the culture vessels without damaging the roots. The culture media was washed away from the roots with running tap water. After that, the plantlets were treated with fungicide (1.5-2 g/l Ridomil) for 20 minutes to prevent unwanted fungal or bacterial growth in the roots (Plate 14). These plantlets were transferred to small polythene bag filled with soil, sand and well decomposed cow dung (1:1:1) and kept in the hardening room for 5-8 days (Plate 15). Then the polythene bags containing plantlets were transferred to net house, where proper care was taken for growth and development of banana plantlets. After 15-20 days, maximum plantlets showed vigorous growth while some were less vigorous (Plate 16). These plantlets were transferred to the field conditions after 30 days (Plate 17) where they grew under conditions of normal environment. The survival rate of plantlets was more than 82 % for both the cultivars.

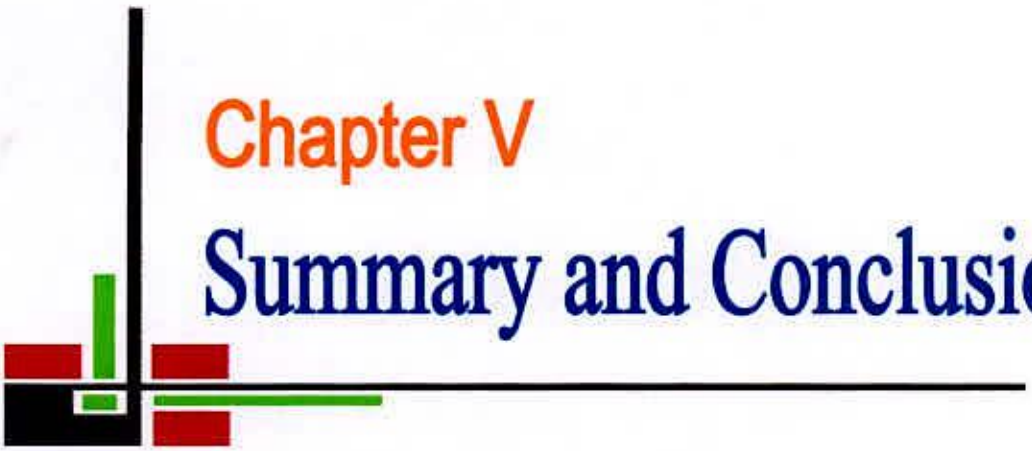
Azad and Amin (1999) reported the same results in *ex vitro* hardening of plantlets. They also monitored the survival (80%) of plantlets under *ex vitro* condition. Cronauer and Krikorian (1984b) observed more or less similar results in *ex vitro* hardening of plantlets.

**Plate 14: *In vitro* plantlets of banana after washing and treated with Ridomil**

**Plate 15: *In vitro* plantlets of banana after transferred to small polythene bag in the hardening room**

**Plate 16: Plantlets of banana under net house after 10 days of transfer to polythene bag**

**Plate 17: Plantlets of banana after 30 days of transfer to polythene bag**



# Chapter V

# Summary and Conclusion

# CHAPTER V

# SUMMARY AND CONCLUSION

The present experiment was conducted with a view to establish a protocol for *in vitro* culture and plant regeneration from shoot tip explants of two cultivars Amritasagar and Sabri of banana (*Musa* spp.) at the laboratory of Proshika Tissue Culture Centre Trust (PTCCT), Manikgonj during the period from April to November, 2008. The experiment was designed following Completely Randomized Design (CRD) with three replications.

Shoot tips excised from field grown plants were used as experimental materials. Explants were sterilized by 0.1% $HgCl_2$ for 14 minutes and shoot tips of both cultivars were found free from contamination after 10 days of sterilization and inoculation and survived cent percent. Treatment, less than 14 minutes period showed *in vitro* contamination and more than 14 minutes treatment, percentage of survival declined considerably. Explants were cultured on MS medium supplemented with different concentrations of BAP (0.0, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0 and 7.0 mg/l) and IBA (0.0, 1.0, 2.0, 3.0, 4.0 and 5.0 mg/l) to assess the influence on *in vitro* shoot regeneration and subsequent root formation. Explants of Amritasagar and Sabri turned brown, light green, green and dark green in colour after 10 days of inoculation but after 20 days all became dark green at all concentrations of BAP except control in Amritasagar and control and 1.0 mg/l in Sabri. In case of single shoot formation, it was observed that the explants on medium at control did not produce single shoot. In Amritasagar, the highest percentage (50%) of explants produced single shoot at 5.0 mg/l BAP, whereas the lowest percentage (20%) of single shoot was produced at 1.0 mg/l BAP. In case of Sabri, the highest percentage (30%) of explants produced single shoot at 5.0 mg/l BAP, whereas the lowest (10%) at 1.0 mg/l BAP. The time required for single shoot regeneration was not similar.



The cultivar Sabri took longer time for single shoot regeneration than Amritasagar. The percentages of survivability and formation of tuber like structure were the highest at 5.0 mg/l among the concentrations of BAP whereas, the lowest at control.

The rate of shoot proliferation varied with cultivars and different concentrations of BAP used. Shoot proliferation rate of Amritasagar was better than that of Sabri. The rate of shoot proliferation increased with the increase of culture period. On the other hand, it increased up to 5.0 mg/l BAP and there after decreased. The cultivar Amritasagar responded more (1.58/explant) on shoot proliferation compared to Sabri (1.00/explant) at 30 days after inoculation. Among the concentrations of BAP, 5.0 mg/l showed the highest shoot proliferation and the lowest was at 1.0 mg/l. No response was observed at control. The cultivar Amritasagar and Sabri produced 3.50 shoots/explant and 2.00 shoots/explant respectively at 5.0 mg/l BAP at 30 days after inoculation.

Shoot length was also influenced by cultivars and different concentrations of BAP used. Among the concentrations of BAP, 5.0 mg/l showed the highest shoot elongation (2.64 cm) in Amritasagar followed by Sabri (2.16 cm) at 30 DAI. The lowest shoot length was found at 1.0 mg/l BAP.

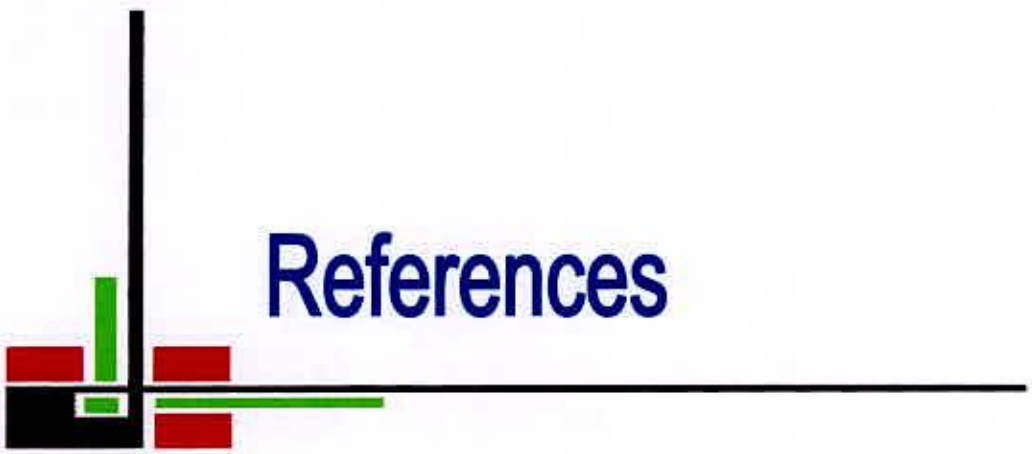
The response of cultivars on leaf formation was found significant. The results showed that Amritasagar produced more leaves (2.35/plantlet) compared to Sabri (1.48/ plantlet) at 30 DAI. In combined effect, 5.0 mg/l showed the highest number of leaves in both the cultivars at different DAI. The cultivar Amritasagar produced 4.11 leaves per plantlet at 5.0 mg/l BAP at 30 DAI, while the Sabri produced 2.67 leaves per plantlet at the same level of BAP and DAI. These results indicated that 5.0 mg/l was the best treatment for leaf formation and Amritasagar was superior to Sabri in respect to formation of leaves per plantlet.

In case of leaf length, Amritasagar responded more compared to Sabri at different DAI. The cultivar Amritasagar and Sabri produced leaf 1.39 cm and 1.08 cm in length respectively at 30 DAI. Among the concentrations of BAP, 5.0 mg/l showed the longest leaf and 1.0 mg/l showed the shortest leaf in both the cultivars at different DAI. The cultivar Amritasagar produced 2.29cm leaf at 5.0 mg/l BAP at 30 DAI, while the Sabri produced 1.86 cm leaf in length.

The number of roots produced per plantlet varied with cultivars and different concentrations of IBA used. The cultivar Amritasagar produced more number of roots compare to Sabri at different DAI. The Amritasagar produced 2.78 roots per plantlet at 30 DAI, while the cultivar Sabri produced 1.80 roots per plantlet at the same DAI. Besides, the number of roots per plantlet increased with culture period. In combined effect, the number of roots produced per plantlet increased with the increase of IBA concentration up to 3.0 mg/l and there after decreased. Among the concentrations of IBA, 3.0 mg/l gave the highest number of roots and 1.0 mg/l gave the lowest number of roots in both the cultivars at different DAI whereas no response was observed in control. The cultivar Amritasagar showed better performance in formation of roots per plantlet (4.10/plantlet) than Sabri (3.63/plantlet) at 3.0 mg/l IBA at 30 DAI. These results indicated that 3.0 mg/l IBA was the best treatment among all the concentrations for formation of roots.

In case of root length, the cultivar Amritasagar produced longer root compared to Sabri at different DAI. The Amritasagar produced roots 2.90 cm in length while the Sabri produced 2.10 cm roots at 30 DAI. On the other hand, IBA at 3.0 mg/l give the highest root length at different DAI in combined effect. The cultivar Amritasagar showed the superior performance in root elongation (3.60 cm) over Sabri (3.10 cm) at 3.0 mg/l IBA at 30 DAI. So, 3.0 mg/l IBA was the best treatment among all the concentrations for increment of root length.

*In vitro* rooted plantlets were successfully acclimatized and transferred to polythene bags containing sand, soil and cowdung (1:1:1). The survival of the plantlets of both cultivars under *ex vitro* condition was more than 82% and all of them resumed growth in the field.



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# Appendices

# APPENDICES

**Appendix I: Composition and concentrations used for the preparation of MS medium (Murashige and Skoog, 1962).**

| Components | Concentrations (mg/l) |
|---|---|
| **Macronutrients** | |
| $KNO_3$ | 1900.00 |
| $NH_4NO_3$ | 1650.00 |
| $MgSO_4.7H_2O$ | 370.00 |
| $CaCl_2.2H_2O$ | 440.00 |
| $KH_2PO_4$ | 170.00 |
| **Micronutrients** | |
| $MnSO_4.4H_2O$ | 22.30 |
| $H_3BO_3$ | 6.20 |
| $ZnSO.7H_2O_4$ | 8.60 |
| $Na_2MoO_4.2H_2O$ | 0.25 |
| $CuSO_4.5H_2O$ | 0.025 |
| $CoCl_2.6H_2O$ | 0.025 |
| KI | 0.83 |
| **Iron Sources** | |
| $FeSO_4.7H_2O$ | 27.80 |
| $Na_2EDTA.\ 2H_2O$ | 37.30 |
| **Vitamins & Organic nutrients** | |
| Nicotine Acid | 0.50 |
| Pyridoxine HCl | 0.50 |
| Thiamine HCl | 0.50 |
| Glycine | 2.00 |
| Myo inositol | 100.00 |
| Sucrose | 30000.00 |
| Agar | 7000.00 |
| pH adjusted to5.8 before autoclaving | |

**Appendix II: Analysis of variance of number of shoots**

| Sources of variation | Degrees of freedom | Mean square of shoot number | | |
|---|---|---|---|---|
| | | 10 DAI | 20 DAI | 30 DAI |
| Factor A (Variety) | 1 | 1.688** | 1.021** | 4.083** |
| Factor B (BAP) | 7 | 0.211* | 1.211** | 4.321** |
| Factor A×B (Variety×BAP) | 7 | 0.209* | 0.211* | 0.321* |
| Error | 32 | 0.083 | 0.093 | 0.135 |

** = Significant at 1% level of probability
* = Significant at 5% level of probability

**Appendix III: Analysis of variance of length of shoots (cm)**

| Sources of variation | Degrees of freedom | Mean square of shoot length (cm) | | |
|---|---|---|---|---|
| | | 10 DAI | 20 DAI | 30 DAI |
| Factor A (Variety) | 1 | 0.032** | 1.714** | 2.284** |
| Factor B (BAP) | 7 | 0.004* | 1.179** | 4.202** |
| Factor A×B (Variety×BAP) | 7 | 0.004* | 0.098** | 0.256* |
| Error | 32 | 0.002 | 0.042 | 0.107 |

** = Significant at 1% level of probability
* = Significant at 5% level of probability

**Appendix IV: Analysis of variance of number of leaves**

| Sources of variation | Degrees of freedom | Mean square of leaf number | | |
|---|---|---|---|---|
| | | 10 DAI | 20 DAI | 30 DAI |
| Factor A (Variety) | 1 | 4.083** | 2.521** | 9.188** |
| Factor B (BAP) | 7 | 0.476** | 3.033** | 8.452** |
| Factor A×B (Variety×BAP) | 7 | 0.476** | 0.676* | 0.876* |
| Error | 32 | 0.036 | 0.270 | 0.269 |

** = Significant at 1% level of probability
* = Significant at 5% level of probability

**Appendix V: Analysis of variance of length of leaves (cm)**

| Sources of variation | Degrees of freedom | Mean square of leaf length (cm) | | |
|---|---|---|---|---|
| | | 10 DAI | 20 DAI | 30 DAI |
| Factor A (Variety) | 1 | 0.021* | 1.209** | 1.175** |
| Factor B (BAP) | 7 | 0.003** | 0.846** | 2.631** |
| Factor A×B (Variety×BAP) | 7 | 0.003** | 0.109* | 0.157* |
| Error | 32 | 0.001 | 0.043 | 0.052 |

** = Significant at 1% level of probability
* = Significant at 5% level of probability

**Appendix VI: Analysis of variance of number of roots**

| Sources of variation | Degrees of freedom | Mean square of root number | | |
|---|---|---|---|---|
| | | 10 DAI | 20 DAI | 30 DAI |
| Factor A (Variety) | 1 | 2.225* | 6.167** | 11.312** |
| Factor B (BAP) | 5 | 1.956** | 7.274** | 16.816** |
| Factor A×B (Variety×BAP) | 5 | 0.339NS | 0.612** | 0.966* |
| Error | 24 | 0.294 | 0.143 | 0.316 |

** = Significant at 1% level of probability
* = Significant at 5% level of probability
NS = Non significant

**Appendix VII: Analysis of variance of length of roots (cm)**

| Sources of variation | Degrees of freedom | Mean square of root length (cm) | | |
|---|---|---|---|---|
| | | 10 DAI | 20 DAI | 30 DAI |
| Factor A (Variety) | 1 | 0.540** | 1.089** | 1.068** |
| Factor B (BAP) | 5 | 0.709** | 1.969** | 2.623** |
| Factor A×B (Variety×BAP) | 5 | 0.066** | 0.050** | 0.064** |
| Error | 24 | 0.017 | 0.009 | 0.016 |

** = Significant at 1% level of probability
* = Significant at 5% level of probability