SOMATIC EMBRYOGENESIS IN *Musa* (AAB) 'NENDRAN'

By

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THESIS

Submitted in partial fulfilment of the requirement for the degree of

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COLLEGE OF HORTICULTURE

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DECLARATION

I hereby declare that this thesis entitled **"Somatic embryogenesis in *Musa* (AAB) 'Nendran'"** is a bonafide record of research work done by me during the course of research and that this thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title of any other University or Society.

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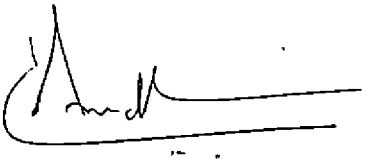
CERTIFICATE

Certified that this thesis, entitled "**Somatic embryogenesis in *Musa* (AAB) 'Nendran'**" is a record of research work done independently by **Miss. Beena, R.** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to her.



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ABBREVIATIONS

BA	-	Benzyl adenine
BAP	-	Benzyl amino purine
°C	-	Degree celsius
cm	-	Centimeter
2,4-D	-	2,4 - dichloro phenoxy acetic acid
EDTA	-	Ethylene diamino tetra acetic acid
GA_3	-	Gibberellic acid
h	-	hour(s)
IAA	-	Indole acetic acid
IBA	-	Indole butyric acid
2ip	-	iso pentenyl adenine
Kin	-	Kinetin, N^6 furfuryl adenine
mg^{-1}	-	milligram(s) per litre
µm	-	Micro molar
MS	-	Murashige and Skoog's (1962) medium
NAA	-	α-Naphthalene acetic acid
pH	-	Hydrogen ion concentration
ppm	-	parts per million
psi	-	Pounds per square inch
uv	-	ultra violet

Introduction

INTRODUCTION

Bananas are considered to be one among the earliest fruits cultivated by man. The oldest reference of banana appears in Ramayana. Scriptures written during the reign of Han dynasty (206 BC - 220 AD) mentioned the cultivation of banana more than 2,000 years ago. It is the staple food crop for people living in many tropical and subtropical countries. In India banana is the most important fruit crop, with an area of 3.96 lakh hectare and a production of 9.925 million tonnes.

Majority of the edible bananas are triploid and are propagated vegetatively by suckers. Sterility and polyploidy often hamper the breeding programmes for the development of superior banana varieties. Plant cell and tissue culture techniques have been developed for the rapid multiplication of elite varieties employing shoot tips and floral apices (Cronauer and Krikorian, 1986). Somatic embryoids have been reported from leaf sheaths, rhizome fragments,from *in vitro* plantlets, thin sections from proliferating buds, immature zygotic embryos, and male flower bud cultures as explant source (Novak *et al.*, 1989; Dhed'a *et al.*, 1991; Marroquin *et al.*, 1993; Escalant *et al.*, 1994; Navarro *et al.*, 1997).

Somatic embryogenesis leading to embryo production and plant regeneration offers distinct advantages for mass propagation of elite cultivars besides being a useful system for genetic manipulation (Israeli *et al.*, 1995; Sagi *et al.*, 1997). Somatic embryos are embryos originating from somatic or vegetative cells, which are very much similar to zygotic embryos having bipolar structure. The development of somatic embryos has been reported by many researchers using

both diploid (Escalant and Teisson, 1988, 1989; Novak *et al.*, 1989; Panis and Swennen, 1993) and triploid (Novak *et al.*, 1989; Dhed'a *et al.*, 1991; Panis and Swennen, 1993; Shii *et al.*, 1992; Escalant *et al.*, 1994) genotypes. Somatic embryogenesis may be used for mass clonal propagation, germplasm handling, cryopreservation and for improving *Musa* spp. through non conventional strategies, i.e., protoplast culture and fusion, mutagenesis, somaclonal variation and genetic transformation. The explant source, media consistency (liquid or solid) as well as organic and inorganic components differ from one author to another.

In banana and plantain, where regeneration follows an embryogenic pathway, somatic embryogenesis may be limited to specific developmental stages. So a detailed study regarding the embryogenic process i.e., which particular cell or tissue develops into embryogenic meristems or at what stage further development stops could provide information for the enhancement or manipulation of the embryogenic response.

The objective of the study on "Somatic embryogenesis in *Musa* (AAB) 'Nendran'" is summarised below.

1) To standardise suitable explant for callus induction in banana (*Musa* AAB) 'Nendran'. The ecotype Manjeri Nendran was utilized for the study. The following explants were tested for callus initiation.

   i. Shoot meristem
   ii. Male flower
   iii. Leaf sheath (scalp)
   iv. Immature embryo

v. Leaf

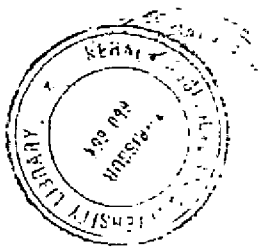
vi. Anther

vii. Root tip

viii. *In vitro* derived root and shoot tips

2) Standardisation of medium for callus regeneration and organogenesis.

The MS medium, modified with respect to macro and micro elements and growth regulators 2,4-D, 2,4,5-T, NAA, BAP, Kinetin etc. to achieve the desired effect.



Review of Literature

REVIEW OF LITERATURE

Banana and plantain are one of the world's most important fruit crop and staple food for millions of people. In India banana contributes 31 per cent of total fruit production.

Annual production of bananas and plantains is estimated to be approximately 88 million tonnes (FAO, 1999) making them one of the largest food crops in the world after rice, maize and wheat (INIBAP, 1997).

The pre history of plant tissue culture began more than 225 years ago. Propagation of plant tissue through tissue culture is based on the concept of cell totipotency derived from the basic cell theory proposed by Schleiden in 1838 and Schwann in 1839.

In recent years remarkable progress has been made in the application of biotechnological methods for mass clonal propagation of several economically important plant species. Biotechnological approaches for the improvement of banana includes cell culture, suspension culture, embryo culture, somatic embryogenesis etc.

The micro propagation technique for cultivated *Musa* is well established (De Guzman *et al.*, 1980; Cronauer and Krikorian, 1984a; Jarret *et al.*, 1985; Vuylsteke and De Langhe, 1985).

2.1 Callus induction studies using different explants

In the study on cell suspension culture system Steward *et al.* (1958) discovered that carrot cells when treated with coconut milk stopped multiplying

and differentiated into immature embryo like structures that were called embryoids. At about the same time Reinert (1959) independently discovered the same phenomenon in carrot grown on agar using high auxin concentration as the inducing agent.

The importance of auxin in culture media for callus induction and for further somatic embryo formation have been reported by many researchers. The explants used for callus induction are usually heterogenous in nature, hence the identification of a suitable explant for each species is the most fundamental step in studies on somatic embryogenesis.

Swamy *et al.* (1983) observed that shoot tips isolated from rhizomes of the banana cultivar Robusta were suitable for production of *in vitro* plantlets.

Several investigators have worked on callus initiation in banana but their effort have met with very limited success. Mohanram and Steward (1964), obtained callus like growth using a number of growth regulators in many preclimateric fruits in their mature and immature stage. Rao *et al.* (1982) have reported that the inflorescence axis can be used as an explant for callus induction. From this callus, roots were also produced but no shoot or bud formation was observed. Geetha *et al.* (1990) have reported the development of *in vitro* callus in black pepper.

When a single shoot was placed on the appropriate semi-solid medium, callus formed at the shoot base. If liquid medium is used, the resulting cell suspension, can be subcultured and they can go on to produce small piece of

nodular callus which in turn go on to produce somatic embryos (Cronauer and Krikorian, 1984b). Bakry and Rossignol (1985) tried various explants of banana for analysing the callus formation and organogenesis and they reported that only floral or inflorescence tissues produced calli but no organogenesis occurred.

There are also reports on the use of shoot tip as explant for callus induction in banana (Jarret *et al.*, 1985; Cronauer and Krikorian, 1986; Huang and Chi, 1988; Dhed'a *et al.*, 1991).

Jarret *et al.* (1985) reported that spherical callus masses were developed from the leaf base explants of banana, which on transfer to a hormone free medium resulted in rapid enlargement and elongation of globular masses but shoot formation from these structures was never observed.

In banana reinitiation of vegetative growth from floral apex explants was reported by many workers (Rao *et al.*, 1982; Rowe, 1984; Cronauer and Krikorian, 1985; Bakry *et al.*, 1985; Balakrishnamoorthy and Sreerangaswamy, 1988; Aravindakshan, 1989; Swamy and Sahijram, 1989; Drew *et al.*, 1989).

Cronauer and Krikorian (1985) observed the reinitiation of vegetative growth from aseptically cultured terminal floral apices of banana. On modified MS medium supplemented with 5 mg l^{-1} BA and 10 per cent coconut water, the determinate floral buds were transformed into multiplying vegetative shoot systems.

A mass of green leafy shoots were observed when the excised floral apices of three banana cultivars, viz., Chandrakele, Rasthali and Robusta on MS

medium when supplemented with cytokinin and auxin. It was also found that the male flower clusters at different stages of development located on the peduncle subtending and distal to the meristematic zone were converted to vegetative state when cultured *in vitro* (Swamy and Sahijram, 1989).

Drew *et al.* (1989) reported that the explant floral apex can be effectively used for multiplying banana plants *in vitro*. Such explants cultured on a medium containing 160 g l^{-1} adenine sulphate, 2 mg l^{-1} IAA and 2 mg l^{-1} kinetin resulted in shoot proliferation.

There are reports on the use of immature embryos as explant for somatic embryogenesis (Cronauer and Krikorian, 1988). Marroquin *et al.* (1993) and Navarro *et al.* (1997) have reported that they used immature zygotic embryo and male flower bud primordia as explant for somatic embryogenesis.

There are also reports on the use of explants like basal leaf sheath and rhizome tissue as explants for callus initiation. Leaf blade can be used as an explant for callus induction and callus development (Kunlayanee-Althachat, 1993). Any part of the banana plant can be used as an explant for somatic embryogenesis but their embryogenic response may vary depending upon the cultivar. Rodriguez and Mendes (1997) used root segments and shoot apices of three banana cultivars taken from *in vitro* cultured plantlets for somatic embryogenesis and found that root segment was a useful explant for banana somatic embryogenesis.

Shoot tip which is the common explant for banana tissue culture can be used as an explant for somatic embryogenesis also (Fitchet-Purnell and Fitchet,

1990). In another study Domingues *et al.*, (1996) used rhizome and pseudostem of banana as explant for somatic embryogenesis.

Even though almost all parts of the banana plant is used as explant for somatic embryogenesis, the most widely used explant is the male flower (Grapin *et al.*, 1996). Escalant *et al.* (1994) have reported the development of somatic embryos from the young flowers of banana and plantain. Gomez-Kosky *et al.* (1994) used male flower as explant for callus induction. Immature male flowers are the useful explants for somatic embryogenesis of banana and plantain (Del-Sol *et al.*, 1995).

In an experiment, conducted to study the somatic embryogenesis of four *Musa* cultivars, using male flower as explant, it was observed that the embryogenic response concentrated in the floral clusters between rows 8 and 13. Here the meristematic nodules formed near vascular elements and from these nodules the embryogenic calli differentiated (Forero, 1995).

In an attempt made by Shchukin *et al.* (1997) to establish a simple and efficient procedure for inducing somatic embryos,they used 3 to 5 leaf primordia and few leaf bases as explant.

Kosky *et al.* (2000) used immature male flower from inflorescences of the hybrid cultivar FHIA-18 (AAAB). Here male flowers were collected directly from the plants at a distance of approximately 20 to 30 cm from the last female flower. The male buds were then cut from the tip and the bracts were removed to give 3 cm length of the basal part of male flower.

The explants when inoculated into the culture media it took about one week for establishment and the established explants can be identified with the slight enlargement of the same.

2.2 Studies on the role of different growth regulators on callus induction

Once the explants got established in the culture media, the next stage is callus formation. The callus formation in plant species was discovered by Duhamel. It was found that the callus formation occurred only in the presence of some specific growth regulators in the culture media. The most important among them being auxin, 2,4-D an auxin, can be used for callus induction effectively (Bakry and Rossignol, 1985). Many reports suggests that MS (Murashige and Skoog) media is more efficient for callus induction, along with 2,4-D (Litz, 1985; Novaille and Petiard, 1988; Dhed'a, 1992; Del-Sol *et al.*, 1995, Navarro *et al.*, 1997) and with NAA and BA (Kunlayanee-Althachat, 1993).

There is another report of callus formation from immature zygotic embryos of diploid banana species using MS medium to which picloram was added and solidified with gelrite (Escalant and Teisson, 1989; Marroquin *et al.*, 1993).

Lee *et al.* (1997) observed that embryogenic calli formed in MS medium having half strength of major nutrients supplemented with 5 μM 2,4-D, 1 μM proline, 100 mg l^{-1} casein hydrolysate.

There are also reports on callus formation in the presence of media containing BAP (Fitchet, 1990) and in SH medium containing Dicamba (Novak *et al.*, 1989).

2.3 Somatic embryogenesis

2.3.1 Role of different growth regulators in Somatic embryogenesis

Somatic embryogenesis is the process by which haploid or diploid somatic cells develop into differentiated plantlets through characteristic embryological stages without the fusion of gametes. The first report on somatic embryogenesis was given by Reinert (1959) in carrot cultures. General pattern of *in vitro* embryogenesis includes direct initiation from different tissues and indirect initiation via callus intermediary. Direct embryogenesis proceeds from embryogenically determined cells (Kato and Kateuchi, 1963). Indirect embryogenesis requires differentiation of the cells, callus proliferation and differentiation of embryogenic cells (Sharp *et al.*, 1980).

Sharp *et al.* (1982) reported the application of somatic embryogenesis for crop improvement. Improved crop varieties can be propagated in large numbers by adopting the standardized protocols of somatic embryogenesis.

Results on somatic embryogenesis were reported by many workers (Sharp *et al.*, 1982; Cronauer and Krikorian, 1983; Banerjee *et al.*, 1985; Fitchet, 1987; Novak *et al.*, 1989; Fitchet-Purnell and Fitchet, 1990; Dhed'a *et al.*, 1991).

Report on somatic embryogenesis had been published by Murashige (1978), Sharp *et al.* (1979), Vasil and Vasil (1980) in various crops. Successful somatic embryogenesis have been reported in many crops like *Coffea arabica* (Sondahl *et al.*, 1979), *Carica papaya* (Litz and Connover, 1982), *Malus pumila* (James *et al.*, 1984), *Oryza sativa* (Raghavan Ram and Nabors, 1984), *Citrus limon* (Carini *et al.*, 1994) and *Elaeis guinensis* (Teixeria *et al.*, 1994).

Somatic embryogenesis can be direct or indirect i.e., callus mediated (Evans *et al.*, 1987). Although callus may be obtained from many species; plant regeneration is possible from some of them only.

It requires an auxin medium for the induction of embryos and a medium devoid of growth regulators for its maturation (Ammirato, 1983). Hussey (1986) also reported that the level of growth regulator in the medium particularly auxin is critical and when this level is lowered there was the chance for embryo formation. In nearly 60 per cent of the tissue cultured plant species where plant regeneration was achieved through somatic embryogenesis, a synthetic auxin, 2,4-D was the only inducer growth regulator. In only a few percentage of tissue cultured plants cytokinins was used either alone or in combination with synthetic auxins to initiate somatic embryogenesis (Evans *et al.*, 1987; Finer, 1994). On the other hand the ability of the cultures to become embryogenic decreased with increase in concentration of 2,4-D. The healthy and organised embryogenic calli when transferred to the medium fortified with high concentration of cytokinin, 2.5 mg l^{-1} and a low concentration of 2,4-D 1.0 g l^{-1} responded rapidly and produced clusters of embryoids within 28 to 30 days (Rout *et al.*, 2000).

Somatic embryo formation was observed after about 2 to 3 months of culture. For somatic embryo formation also auxin plays an important role. In many cases somatic embryos formed in presence of an auxin like 2,4-D (Cronauer and Krikorian, 1988; Ma *et al.*, 1989; Navarro *et al.*, 1997). 2,4,5-T (Cronauer and Krikorian, 1983) and NAA along with picloram (Escalant and Teisson, 1989) with 2,4-D, proline, CH and ascorbic acid (Perez *et al.*, 1998) and NAA, L-glutamine, L-proline, kinetin, zeatin (Kosky *et al.*, 2000).

Jarret *et al.* (1985) reported the formation of spherical callus masses resembling globular structures in presence of Dicamba in MS medium. MS media containing zeatin also gave rise to somatic embryos (Novak *et al.*, 1989).

2.3.2 Role of different explants on Somatic Embryogenesis

The somatic embryos appeared as small swellings which resembled zygotic embryos (Jarret *et al.*, 1985) or they formed on the surface of a translucent callus which formed over the yellow callus (Del-Sol *et al.*, 1995) and it took 4 to 6 months for somatic embryos to develop.

Rhizome explants of triploid banana cultivar Grand Naine also gave rise to somatic embryos (Lee *et al.*, 1997).

Another route by which somatic embryos are formed is by protoplast culture, without any callus phase (Panis and Swennen, 1993; Cote *et al.*, 1996).

The studies conducted using different explants reveals that somatic embryos can be successfully induced from explants like male flowers and immature embryos (Navarro et al. 1997).

2.3.3 Somatic embryo maturation and germination

After formation it takes some time for the maturation of somatic embryos. The growth regulators that are needed for maturation phase is different, if auxins are essential for callus and somatic embryo formation, for somatic embryo maturation the medium should be devoid of auxin (Novaille and Petiard, 1988) and they need the presence of cytokinin in the media. Cytokinins like BA or zeatin favoured the maturation and germination of somatic embryos (Dhed'a *et al.*, 1991) and in the presence of BAP also the same effect was observed (Kosky *et al.*, 2000). The presence of zeatin and kinetin in the culture media helped somatic embryo maturation (Navarro *et al.*, 1997).

Apart from the presence of growth regulators some growth retardant like abscicic acid also favour somatic embryo maturation (Grapin *et al.*, 1996).

Litz (1985) reported that the germination of somatic embryos of tropical fruit trees has been difficult to demonstrate with any consistency. However, many scientists got positive results regarding the germination of somatic embryos.

Cronauer and Krikorian (1988) reported that the somatic embryos germinated by transferring them to basal SH medium and then to basal MS medium then they produced the first green leaves. There are reports on somatic embryo germination in medium containing NAA (Escalant and Teisson, 1989). Benzyl adenine and gibberellin (Ma *et al.*, 1989).

Novaille and Petiard (1988) reported the germination of somatic embryos on half MS medium containing zeatin alone or zeatin along with ascorbic acid (Perez *et al.*, 1998). The most effective growth regulator for somatic embryo germination were cytokinins like BAP (Fitchet, 1990; Megia *et al.*, 1993), BA along with an auxin IAA (Navarro *et al.*, 1997) or BA and picloram (Megia *et al.*, 1993).

Another report shows that the somatic embryos germinated in presence of gibberellin (Pancholi *et al.*, 1995). Here it was found that the addition of gibberellin increased shoot and root number.

There are also reports, which say that even though somatic embryos are formed their germination is difficult (Domingues *et al.*, 1996).

Many reports on somatic embryogenesis shows that, even if normal root formation is there, no shoot formation was observed (Cronauer and Krikorian, 1984b; Jarret *et al.*, 1985; Lee *et al.*, 1997; Fitchet-Purnell and Fitchet, 1990).

Materials and Methods

MATERIALS AND METHODS

The present study on "Somatic embryogenesis in *Musa* (AAB) 'Nendran'", was carried out in the Tissue Culture Laboratory, Department of Pomology and Floriculture, College of Horticulture during 1998-2000.

In the research programme, plant regeneration was attempted using explants from the banana variety Nendran (*Musa* (AAB) 'Nendran') through a callus phase and somatic embryogenesis. The ecotype Manjeri Nendran was the material for the study. The first part of the experiment consisted of standardisation of suitable explant for callus induction. The different stages of the experiment are given below.

3.1 Selection of explant

For callus induction and somatic embryogenesis the following explants were tried; namely, shoot meristem, male flower, leaf sheath (scalps), immature embryo, leaf, anther, root tip and *in vitro* derived root and shoot tips.

3.1.1 Collection and preparation of explants

The explants were collected from the field grown plants on the same day of inoculation.

For shoot tip explants the main apical buds were collected from five month old sword suckers of banana. Suckers after separation were detopped, and reduced to a size which measured about 1.5 cm in length retaining a small portion of the rhizome tissue. In the case of male flower, they were collected after the completion of female phase. The bracts of the flowers were removed leaving about three cm of the basal part.

The other explants tested for callus induction include, leaf, leaf sheath, root, anther, *in vitro* root and shoot tips. Explants were collected both from the *in vitro* and *ex vitro* grown plants and from the mature and immature stages of the plant.

The explants collected from field grown plants were then subjected to surface sterilization procedures. They were washed thoroughly to remove the dirt and adhering soil particles. In the case of leaf, leaf sheath, root and immature embryo after washing with tap water, they were wiped with 70 per cent alcohol. Then the explants were given an antibiotic and fungicide treatment to reduce the contamination caused by bacteria and fungi respectively and further sterilization procedures were carried out under perfect aseptic conditions in a Klenzoids laminar air flow cabinet. The surface sterilization treatments given for different explants are given in Table 1.

As the explants collected from *in vitro* grown plantlets were under aseptic condition, no surface sterilization was given.

For shoot meristem and male flower, first they are washed with tap water, treated with an antibiotic Norfloxacin 0.1 per cent for 30 minutes followed by treatment with a seed dressing fungicide Emisan 0.1 per cent for 15 minutes. In case of leaf as explant it was first washed in tap water, then wiped with 70 per cent alcohol and treated with Emisan at 0.1 per cent concentration for 15 minutes. It was then followed by sterilization with $HgCl_2$ 0.1 per cent concentration for five minutes.

The other explants used in this experiment namely, leaf sheath, immature embryo, root and anther were first washed with tap water, wiped with 70

per cent alcohol followed by treating with antibiotic Norfloxacin 0.1 per cent concentration for 25 minutes, then treated with Emisan 0.1 per cent concentration for 10 minutes and finally with $HgCl_2$ 0.1 per cent concentration for three minutes.

The explants after surface sterilization were rinsed three times with sterile distilled water and then dried by carefully transferring them on to sterile filter paper placed over a sterile petri dish.

Table 1. Surface sterilization treatments for different explants of banana

Explant	Sterilant	Conc. (%)	Duration
Shoot meristem and male flower	Norfloxacin Emisan $HgCl_2$	0.1 0.1 0.1	30 minutes 15 minutes 5 minutes
Leaf	Emisan $HgCl_2$	0.1 0.1	15 minutes 5 minutes
Leaf sheath Immature embryo Root Anthers	Norfloxacin Emisan $HgCl_2$	0.1 0.1 0.1	25 minutes 10 minutes 3 minutes

The sterilized explants were further reduced in size by trimming down the cut surface of the explants under sterile condition. Inflorescence explants were sliced into pieces of about 0.5 cm length and 1.0 cm breadth before inoculation. In the case of male flower after sterilization treatments the bases of the flowers were excised from the other parts. In case of leaf, sections of about five mm were isolated and inoculated into the culture medium.

After preparation, all the explants were cultured into appropriate media.

3.2 Preparation of media

The culture medium used for the study was MS (Murashige and Skoog, 1962) supplemented with different growth regulators. The MS medium varying in its concentration of macro nutrients were tried in order to find out a superior medium for callus induction and for its further proliferation. The chemical composition of the media is given in Table 2.

Table 2. Composition of chemicals for the preparation of MS stock solution

Chemicals	Quantity ($g\ l^{-1}$)	Concentration	Volume to be pipetted for one litre media
NH_4NO_3	82.5	50x	For full MS 20 ml For half MS 10 ml For quarter MS 5ml
KNO_3	96		
KH_2PO_4 stock I	8.5		
$MgSO_4.7H_2O$	18.5		
$CaCl_2.2H_2O$ (stock II)	22	50 x	For full MS 20 ml For half MS 10 ml For quarter MS 5ml
Na2 EDTA stock III	3.7	100 x	,,
$FeSO_4.7H_2O$	2.8		
$MnSO_4.4H_2O$	2.23	100x	,,
$ZnSO_4.7H_2O$	0.86		
H_3BO_3	0.62		
KI stock IV	0.083		
$Na_2M_0O_4.2H_2O$	0.025		
$CoCl_2\ 6H_2O$	0.0025		
$CuSO_4\ 5H_2O$	0.0025		
Glycine	0.2	100 x	,,
Pyridoxine	0.05		
Nicotinic acid stock V	0.05		
Thiamine	0.01		
Myo inositol	0.1	-	-
Sugar	30	-	-
Agar	7	-	-
pH 5.7	-	-	-

Stock solutions were prepared in order to make the media preparation procedure easy. Five stock solutions were prepared. The stock solution I containing

four chemicals (NH_4NO_3, KNO_3, KH_2PO_4 and $MgSO_4.7H_2O$). They were weighed separately and dissolved in 500 ml distilled water one by one in the above mentioned order and made upto one litre with distilled water. The stock solution II contained only one chemical, $CaCl_2.2H_2O$. The stock solution III was prepared by dissolving Na_2EDTA and $FeSO_4.7H_2O$ in separate beakers with 200 ml distilled water, both the beakers were placed on hot plates when it started boiling, $FeSO_4.7H_2O$ was added to Na_2EDTA (15 minutes with continuous stirring) cooled to room temperature, made up to one litre with distilled water. The stock solution IV was prepared by dissolving the seven micro nutrients ($MnSO_4.4H_2O$, $ZnSO_4.7H_2O$, H_3BO_3, KI, $Na_2MoO_4.2H_2O$, $CoCl_2.6H_2O$ and $CuSO_4.5H_2O$) in 500 ml of water and later made up to one litre with distilled water.

The stock solution V contained the essential vitamins necessary for growth and development. While preparing media, for dissolving the growth regulators belonging to auxin group they were first dissolved in minimum quantity of weak basic (10 N NaOH) solutions and cytokinins in weak acidic (10 N HCl) solutions and made up with distilled water.

The stock solutions I and II were prepared to 50 x concentration while stock solutions III, IV and V at 100 x concentration. Using this, to prepare one litre of full MS media, the stock solutions were pipetted at a volume of 20:20:10:10:10 milli litres respectively. To this myo-inositol was added @ 0.1 g l^{-1} and sugar at 30 g l^{-1}.

For MS media, the favourable pH is 5.7. This pH was adjusted by adding either 0.1 N NaOH or HCl. After adjusting the pH, agar @ 7 g l^{-1} was added.

The media was then boiled for melting the agar. It was then poured hot to test tubes which were previously rinsed with double distilled water and oven-dried. The test tubes were then tightly plugged with sterilized cotton wool plugs.

In order to ensure aseptic condition of the medium, the medium in the test tubes were autoclaved for 15 to 20 minutes at 15 psi pressure and 121°C temperature (Dodds and Roberts, 1985). After sterilization, the culture vessels were immediately transferred to the culture room.

3.3 Inoculation of explants

All the inoculation operations were carried out in a 'Klenzoids' Laminar flow cabinet under perfect aseptic conditions.

To inoculate the explants on the culture medium, the cotton wool plug of the culture vessel was removed and the neck of the test tube was first flamed over a LPG burner in the chamber. The sterile explants were quickly transferred into the medium using sterilized forceps, then once again the neck of the test tube was flamed and the cotton wool plug quickly replaced.

The test tubes were then transferred to a culture room where they were incubated at a temperature of 27±2°C. Artificial illumination was provided using tube lights, such a way that the light intensity was maintained at 2000 lux for 16 hours.

3.4 Callus induction

The MS medium containing the basal salts (Macro & micro nutrients) vitamins, myo inositol, sucrose and various concentrations of different growth regulators were used for callus induction. The treatment combinations attempted are given in Table 3.

Table 3. The different treatment combinations to find out the effect of different growth regulators on different explants of *Musa* (AAB) Nendran on callus induction

Explants	Treatments
Shoot meristem, male bud, leaf sheaths (scalps), immature embryos, leaves, anthers, root tips, *in vitro* derived shoot and root tip	3 x 3 combinations of full MS, half MS, quarter MS and 2, 4-D (0.5, 1.0, 2.0) ppm, NAA (1.0, 2.5, 5.0) ppm, 2, 4, 5-T (0.25, 0.5, 1.0) ppm
Male flower	3x3 combination of 2,4-D (0.5, 1.0, 2.0) ppm and BAP (1.0, 2.0, 4.0) ppm
Male flower and immature embryos	3x3 combination of 2, 4, 5-T (0.25, 0.5, 1.0) ppm & BAP (0.5, 1.0, 2.0) ppm 2,4-D (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9) ppm 2, 4, 5-T (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9) ppm

Observation on the number of days taken for culture establishment and callus induction as well as the callusing percentage and callus index were worked out at 30, 45 and 60 days after inoculation.

3.4.1 Callus index

The callus index was calculated using the formula

$CI = P \times G$

Where P = Percentage of callus inititation

G = Growth score

Scoring was done based on the spread of the calli and a maximum score of four was given for those that have occupied the whole surface of the media within 60 days of culture period in culture tubes.

3.5 Callus proliferation

The MS basal medium supplemented with various growth regulators were tried for callus proliferation.

Table 4. Different treatment combinations tried for callus proliferation

Explant	Treatment
Calli obtained from male flower explants	4x3 combination of BAP (1.0, 2.0, 3.0, 4.0) ppm and NAA (1.0, 2.0, 3.0) ppm 2, 4-D (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0) ppm 3 x 4 combination of 2, 4-D (0.5, 1.0, 2.0,) ppm and BAP – (0.5, 1.0, 2.0, 4.0) ppm NAA (0.1, 0.2, 0.3, 0.4, 0.5) ppm Kinetin (0.1, 0.2, 0.3, 0.4, 0.5) ppm

Observations were taken 30 day after subculturing on percentage of cultures showing callus proliferation.

3.6 Indirect organogenesis

Table 5. Different treatment combinations tried for indirect organogenesis from the calli obtained from the explants of *Musa* (AAB) 'Nendran'

Explant	Treatment
Calli maintained in MS medium supplemented with 2,4-D 0.5 ppm	NAA (0.1, 0.2, 0.3, 0.4, 0.5) ppm Kinetin (0.1, 0.2, 0.3, 0.4, 0.5) ppm 2, 4-D (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.5, 2.0) ppm 3 x 3 combination of 2,4-D (0.5, 1.0, 2.0) and BAP (0.25, 0.5, 1.0) ppm GA_3 (0.1, 0.5, 1.0, 1.5) ppm 4 x 1 combination of GA_3 (0.1, 0.5, 1.0, 1.5, 2.0, 4.0, 6.0) ppm and BAP 4.0 ppm 4 x 3 combination of BAP (1.0, 2.0, 3.0, 4.0) ppm and NAA (1.0, 2.0, 3.0) ppm

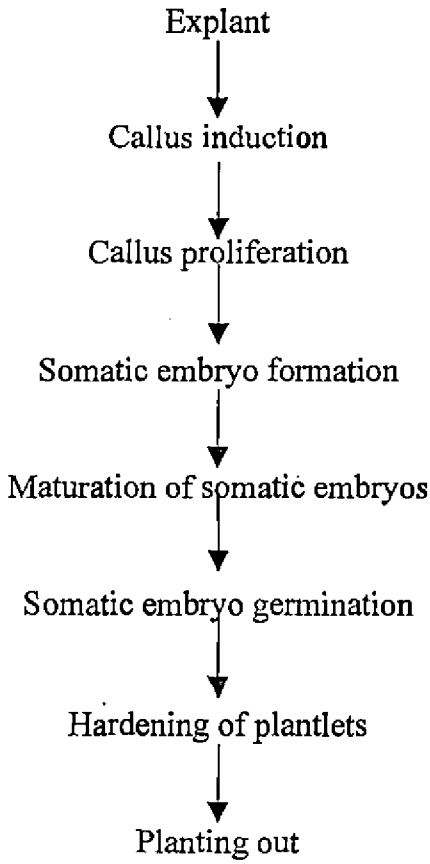
3.7 Somatic embryogenesis

Table 6. Different treatment combinations tried for somatic embryogenesis in *Musa* (AAB) 'Nendran'

Explant	Treatment
Calli maintained in MS medium supplemented with 2,4-D 0.5 ppm.	Full MS with 2,4-D (0.0, 0.1, 0.5, 1.0, 1.5) ppm Full MS with 4x3 combination of BAP (1.0, 2.0, 3.0, 4.0) ppm and NAA (1.0, 2.0, 3.0) ppm. Half MS with 2,4-D (0.5, 1.0, 1.5) ppm. Half MS with 4x3 combinations of BAP (1.0, 2.0, 3.0, 4.0) ppm and NAA (1.0, 2.0, 3.0) ppm.
Calli obtained from male flowers	2,4-D (0.5, 1.0, 2.0) ppm 4x3 combinations of BAP (1.0, 2.0, 3.0, 4.0) ppm and NAA (1.0, 2.0, 3.0) ppm. 3x2 combinations of kinetin (0.5, 1.0, 2.0) ppm and NAA (0.5, 1.0) ppm. BAP (1.0, 2.0, 3.0, 4.0) ppm.

All the experiments for somatic embryogenesis were carried out using full strength MS medium. Those explants which were responding well to callus induction and callus proliferation were used as explant for somatic embryogenesis. The different stages involved in somatic embryogenesis of banana is presented in Fig.1.

Fig.1. Route of somatic embryogenesis in Banana



Results

RESULTS

The results of the investigations on somatic embryogenesis in *Musa* (AAB) 'Nendran' conducted during 1998-2000 in the Tissue Culture Laboratory, Department of Pomology and Floriculture, College of Horticulture are presented in this chapter.

4.1 Callus induction

4.1.1 Standardisation of media and explant

In order to find out a suitable concentration of MS medium and explant for callus induction using different explants of banana, the MS (Murashige and Skoog, 1962) medium having three concentrations of macro nutrients i.e. full MS, half MS and quarter MS were tried using three different growth regulators viz., 2,4-D, 2,4,5-T and NAA. The data are presented in Table 7, 8 and 9. From the observations recorded it was found that all the concentration of 2,4-D and 2,4,5-T tried were capable of inducing atleast slight callus in almost all the explants cultured in all the three concentrations of MS medium. NAA showed response only at 1.0 ppm concentration, that too in full MS medium.

In 2,4-D at 0.5 ppm, the results were better compared to all other treatments of 2,4-D and 2,4,5-T. In general 2,4-D was found to be the best growth regulator for callus induction in all the explants tried. However, the callus inducing property of 2,4-D was reduced with the increase in concentration. 2,4-D induced early callusing in almost all the explants (14 to 30 days) (Table 7).

Table 7. Effect of 2,4-D with different levels of MS medium on callus induction using different explants of *Musa* (AAB) 'Nendran'

Treat-ment	Explant	Callusing %												Callus morphology		
		full MS (a)				Half MS (b)				quarter MS (c)						
		30 DAI	45 DAI	60 DAI	Callus index 60 DAI	30 DAI	45 DAI	60 DAI	Callus index 60 DAI	30 DAI	45 DAI	60 DAI	Callus index 60 DAI	a	b	c
2,4-D 0.5 ppm	Leaf	40.00	46.60	53.30	119.93	23.30	50.00	50.00	73.99	20.00	36.67	36.67	43.28	Compact, green calli no regenerative capacity	White compact calli	Green calli
„	*In vitro* shoot	23.33	36.67	36.67	80.01	20.10	40.40	40.40	60.00	26.67	43.33	43.33	46.66	Compact, green calli	White friable calli	Slight bulging of explants
„	Leaf sheath	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	No callus induction	No callusing	No callus
„	Root	0.00	0.00	0.00	0.00	13.30	13.30	13.30	13.30	0.00	6.00	10.00	10.00	„	Slight bulging of explants	Slight brownish calli
„	Anther	63.30	76.70	76.70	246.77	50.00	83.33	83.30	72.00	30.00	60.00	63.33	69.99	White, friable calli with regenerative potential	White friable calli	White friable calli
„	Meristem	0.00	50.00	50.00	100.00	16.67	26.60	38.60	48.75	0.00	16.60	16.60	23.24	Compact callusing, white with no regeneration potential	Slight brownish calli	Brownish callus
„	Immature embryo	13.30	33.30	63.30	243.20	60.00	73.30	73.30	99.54	23.30	40.00	40.00	53.33	White, friable calli with regeneration potential	White compact calli	White, compact calli
„	Male flower	50.00	60.00	96.67	228.75	26.67	40.00	74.00	112.50	26.67	43.33	43.33	66.67	Cream, friable calli with regeneration potential	White friable callus	White friable calli
Mean		23.74	37.9	47.08	127.33	26.25	40.86	46.61	60.01	15.83	30.74	31.65	39.14			

Contd.

Table 7. Continued

Treat-ment	Explant	Callusing %												Callus morphology		
		full MS (a)				half MS (b)				quarter MS (c)						
		30 DAI	45 DAI	60 DAI	Callus index 60 DAI	30 DAI	45 DAI	60 DAI	Callus index 60 DAI	30 DAI	45 DAI	60 DAI	Callus index 60 DAI	a	b	c
2,4-D 1.0 ppm	Leaf	26.70	36.60	40.00	53.33	23.34	36.67	36.67	53.33	20.00	43.33	43.33	53.32	Very slight callusing, compact calli, green	Greenish compact calli	Greenish compact calli
,,	*In vitro* shoots	26.70	28.30	28.30	49.96	16.67	23.33	23.33	26.66	6.00	33.33	36.67	44.00	Slight callusing	Greenish compact calli	Greenish compact calli
,,	Leaf sheath	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	No callusing	White friable calli	No callusing
,,	Root	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	20.00	20.00	20.00	20.00	No callusing	Brownish calli	Slight brownish calli
,,	Anther	33.00	60.00	60.00	159.90	40.00	53.33	53.33	126.65	30.00	56.67	56.67	80.00	White friable calli	White compact calli	White friable calli
,,	Meristem	0.00	33.30	33.33	66.67	26.66	33.33	33.33	39.99	0.00	26.67	33.33	36.70	No callusing	White friable calli	White calli
,,	Immature embryo	13.30	20.00	40.00	148.00	13.33	33.33	40.00	46.67	16.67	26.67	26.67	33.34	White friable calli		White compact calli
,,	Male flower	40.00	46.00	53.00	156.00	16.67	20.00	26.60	46.67	23.30	36.67	36.67	43.33	White friable calli		White friable calli
Mean		17.46	28.02	31.83	79.23	17.08	24.99	26.65	42.49	14.49	30.41	31.66	38.83			

Contd.

Table 7. Continued

Treat-ment	Explant	Callusing %												Callus morphology		
		full MS (a)				half MS (b)				quarter MS (c)						
		30 DAI	45 DAI	60 DAI	Callus index 60 DAI	30 DAI	45 DAI	60 DAI	Callus index 60 DAI	30 DAI	45 DAI	60 DAI	Callus index 60 DAI	a	b	c
2,4-D 2.0 ppm	Leaf	13.30	23.40	23.50	30.08	10.00	23.33	23.33	29.9	23.33	46.67	46.67	50.00	Compact, green calli no regenerative capacity	Slight callusing, brownish calli	Slight brownish calli
„	*In vitro* shoots	20.00	33.00	33.00	42.90	0.00	6.00	23.33	26.62	16.67	40.00	40.00	43.33	Compact, green calli	Slight greenish compact calli	Slight brownish calli
„	Leaf sheath	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	No callus induction	White, friable calli	No callusing
„	Root	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	„	Slight white calli	No callusing
„	Anther	6.67	30.00	43.00	86.67	16.67	30.00	30.00	36.67	0.00	16.67	16.67	23.34	White, friable calli with regenerative potential	White compact calli	White friable calli
„	Meristem	0.00	26.67	26.67	53.34	0.00	0.00	20.00	20.00	0.00	23.33	23.33	26.66	Compact callusing, white with no regeneration potential	White, friable calli	Brownish calli
„	Immature embryo	16.70	30.00	30.00	165.59	26.67	43.33	43.33	56.66	13.30	23.30	23.30	34.95	White, friable calli with regeneration potential		White, compact calli
„	Male flower	16.70	33.40	43.30	100.71	23.33	36.66	36.66	56.65	16.66	26.67	26.67	40.01	Cream, friable calli with regeneration potential		White friable calli
Mean		9.17	22.06	24.93	59.91	9.58	17.41	22.08	28.31	8.74	22.08	22.08	27.28			

2,4,5-T induced callusing in majority of the explants but the callus inducing property in different explants were much delayed. It took 20 to 60 days for callus induction in various explants. Here also the callus inducing properties reduced with increasing concentrations of 2,4,5-T (Table 8).

Among the different explants tried for callus induction, the anther produced profuse callusing in many of the treatment followed by the male flower and immature embryo. The leaf sheath did not produce any callus in any treatment. The root produced slight callus when cultured in media containing 2,4-D (0.25, 0.5 and 1.0 ppm) and 2,4,5-T (0.5 and 1.0 ppm) but the response was very negligible and further increase in callusing were not noticed. The male flower responded better in 2,4-D while immature embryo produced good response in 2,4,5-T. Even though the leaf cutting and shoot meristem produced slight callusing, their callusing percentage and callus index were very low in all the treatments.

Regarding the time taken for the culture establishment and callus induction, it varied according to various growth regulators and explants used. The anthers produced callusing within 14 days when 2,4-D was used, followed by male flower and immature embryo, which produced callus within a period of 18 to 24 days. The leaf sheath did not produce any callus even after 60 days, the leaf and root produced calli within 30 days. When 2,4,5-T was used, the anther exhibited callusing within 25 days while the immature embryo and male flower showed callusing within 30 to 52 days, the root and leaf took more than 55 days for callus initiation. Here also the leaf sheath did not produce any callusing.

Table 8. Effect of 2,4,5-T with different levels of MS medium on callus induction using different explants of *Musa* (AAB) 'Nendran'

Treat-ment	Explant	Callusing %												Callus morphology		
		full MS (a)				half MS (b)				quarter MS (c)						
		30 DAI	45 DAI	60 DAI	Callus index 60 DAI	30 DAI	45 DAI	60 DAI	Callus index 60 DAI	30 DAI	45 DAI	60 DAI	Callus index 60 DAI	a	b	c
2,4,5-T 0.25 ppm	Leaf	6.67	36.67	40.00	56.00	30.00	40.00	43.30	49.99	20.00	33.33	40.00	43.33	Slight calm, white compact	Greenish calli	Slight greenish calli
,,	*In vitro* shoots	10.00	33.30	43.30	46.63	20.00	26.67	26.67	33.33	6.00	26.66	26.66	33.32	Greenish compact calli	Greenish calli	Slight greenish calli
,,	Leaf sheath	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	No callusing	No callusing	No callusing
,,	Root	16.00	0.00	0.00	16.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	Slight compact brownish callus	No callusing	No callusing
,,	Anther	26.60	60.00	66.67	150.01	6.00	53.30	60.00	129.00	16.67	23.33	23.33	53.33	White friable callus	White friable non regenerative callus	White friable calli
,,	Meristem	0.00	0.00	40.00	40.00	0.00	13.30	30.00	36.66	0.00	0.00	16.67	20.01	Brownish compact calli	White friable calli	Slight white calli
,,	Immature embryo	6.70	46.67	56.66	66.65	10.00	40.00	70.00	79.99	0.00	30.00	43.33	56.67	White compact calli	White compact calli	White compact calli
,,	Male flower	10.00	33.30	46.67	56.67	20.00	50.00	56.60	66.58	10.00	23.33	23.33	36.67	White friable calli	White friable calli	White friable calli
Mean		9.49	26.24	36.66	53.99	10.75	27.90	35.82	49.44	6.58	17.08	21.66	30.35			

Contd.

Table 8. Continued

Treat-ment	Explant	Callusing %												Callus morphology		
		full MS (a)				half MS (b)				quarter MS (c)						
		30 DAI	45 DAI	60 DAI	Callus index 60 DAI	30 DAI	45 DAI	60 DAI	Callus index 60 DAI	30 DAI	45 DAI	60 DAI	Callus index 60 DAI	a	b	c
2,4,5-T 0.5 ppm	Leaf	13.30	40.00	40.00	53.33	10.00	30.00	40.00	50.10	13.33	23.34	23.34	36.70	Greenish compact calli	Greenish compact calli	Greenish calli
,,	*In vitro* shoots	16.67	33.30	33.30	56.61	20.00	46.66	46.60	53.32	0.00	16.67	30.00	33.30	Slight callusing, white compact	Greenish compact calli	Greenish calli
,,	Leaf sheath	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	No callusing	No callus	No callusing
,,	Root	0.00	0.00	10.00	20.00	0.00	0.00	6.00	6.00	0.00	0.00	3.00	3.00	Brownish compact calli	Brownish calli	Very little brownish calli
,,	Anther	23.30	50.00	60.00	86.40	21.10	66.60	66.60	81.39	20.00	36.67	36.67	46.67	Brownish calli friable with no regeneration potential	White friable calli	White friable calli
,,	Meristem	0.00	0.00	26.60	26.60	0.00	40.00	53.30	63.29	0.00	13.33	20.00	26.67	Slight white callusing	Brownish calli	Brownish calli
,,	Immature embryo	16.67	30.00	30.00	36.60	6.00	33.30	40.00	50.00	0.00	33.33	35.00	66.67	White friable callusing with no regeneration potential	White calli	White compact calli
,,	Male flower	13.30	26.67	36.67	46.67	0.00	10.00	30.00	43.30	23.30	43.33	43.33	59.99		Slight white calli	White friable calli
Mean		10.40	22.49	29.57	40.77	7.15	28.32	35.31	43.42	7.07	25.83	23.91	34.12			

Contd.

Table 8. Continued

Treat-ment	Explant	Callusing %												Callus morphology		
		full MS (a)				half MS (b)				quarter MS (c)						
		30 DAI	45 DAI	60 DAI	Callus index 60 DAI	30 DAI	45 DAI	60 DAI	Callus index 60 DAI	30 DAI	45 DAI	60 DAI	Callus index 60 DAI	a	b	c
2,4,5-T 1.0 ppm	Leaf	0.00	43.30	43.30	40.96	0.00	23.30	53.30	59.96	0.00	16.67	26.67	36.67	Slight bulging of explants	Greenish compact calli	Greenish calli
„	*In vitro* shoots	46.60	60.00	60.00	66.70	0.00	16.60	30.00	30.00	10.00	20.00	20.00	26.66	Slight callusing	Greenish compact calli	Greenish calli
„	Leaf sheath	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	No callusing	No callus	No callusing
„	Root	0.00	0.00	16.60	33.20	0.00	0.00	6.00	12.00	0.00	0.00	10.00	10.00		Brownish calli	Brownish calli
„	Anther	26.70	50.00	60.00	69.90	20.00	36.67	36.67	46.67	16.67	43.30	43.30	49.96		White friable calli	White friable calli
„	Meristem	10.00	23.30	23.30	26.62	0.00	10.00	26.60	29.93	0.00	10.00	23.30	29.96		Brownish calli	White calli
„	Immature embryo	6.00	33.40	40.00	46.67	10.00	23.30	53.30	56.63	0.00	26.67	36.60	39.93		White calli	White compact calli
„	Male flower	20.00	36.67	46.67	60.01	16.00	50.00	61.10	61.10	20.00	33.33	33.33	49.99		Slight white calli	White friable calli
Mean		13.66	30.83	36.23	43.00	5.75	19.98	33.37	37.03	5.83	18.74	24.15	30.39			

Table 9. Effect of NAA with different levels of MS medium on callus induction using different explants of *Musa* (AAB) 'Nendran'

Treat-ment	Explant	Callusing %												Callus morphology		
		full MS (a)				half MS (b)				quarter MS (c)						
		30 DAI	45 DAI	60 DAI	Callus index 60 DAI	30 DAI	45 DAI	60 DAI	Callus index 60 DAI	30 DAI	45 DAI	60 DAI	Callus index 60 DAI	a	b	c
NAA 1.0 ppm	Leaf	16.67	26.67	26.67	26.67	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	Slight bulging of explant	-	-
,,	*In vitro* shoots	20.00	33.30	33.30	33.30	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	,,	-	-
,,	Leaf sheath	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	No callus was noticed even after 30 days	-	-
,,	Root	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	,,	-	-
,,	Anther	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	,,	-	-
,,	Meristem	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	,,	-	-
,,	Immature embryo	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	,,	-	-
,,	Male flower	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	,,	-	-
Mean		4.58	7.49	7.49	7.49	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00			

NAA 2.5 ppm and NAA 5 ppm did not produce any result in any treatment combination

When NAA was used at 1.0 ppm in full MS medium, slight bulging was observed. Higher concentrations of NAA showed an inhibitory effect on callusing. The other explants used in the experiment did not produce callus in any of the three concentrations of MS media supplemented with different concentrations of NAA (Table 9).

Regarding the nature of calli produced, the anther and male flower produced white, friable, loose calli while the immature embryo produced white compact calli, the leaf and *in vitro* shoot produced slight, compact greenish calli. The shoot meristem produced white compact calli without regeneration potential. The calli produced by male flowers only exhibited regeneration potential.

4.1.2 Effect of concentration of basal media

The observation on the effect of three concentrations of basal media on various explants is given in Table 10. From the data it was found that 30 days after inoculation the callus induction recorded with respect to different explants cultured in three different MS media containing same concentration of growth regulators i.e. 2,4-D 0.5 ppm recorded varying results. The maximum callus induction was noticed in full MS medium (41.65), which is closely followed by half MS medium (40) and the quarter MS medium recorded the least value (24.99). When 2,4,5-T was used at 0.5 ppm concentration, the maximum callus induction after 30 days of inoculation were noticed with full MS medium (16.64) followed by quarter MS (14.15). The half MS medium recorded the lowest value (11.77).

4.1.3 Combined effect of auxins with cytokinins

The effect of combination of 2,4-D and BAP on callus induction from male flower explants is given in Table 11. Maximum callusing percentage

Table 10. Response of different explants in different basal media supplemented with 2,4-D at 0.5 ppm and 2,4,5-T at 0.5 ppm for callus induction in *Musa* (AAB) 'Nendran'

Explants	Full MS + 2,4-D 0.5 ppm			Half MS + 2,4-D 0.5 ppm			Quarter MS + 2,4-D 0.5 ppm		
	Callusing % DAI			Callusing % DAI			Callusing % DAI		
	30	45	60	30	45	60	30	45	60
Leaf	40.00	46.60	53.30	23.30	50.00	50.00	20.00	36.67	36.67
Male flower	50.00	60.00	96.70	26.70	40.00	74.00	26.67	43.33	53.34
Anther	63.30	76.70	76.70	50.00	83.30	83.30	30.00	60.00	63.33
Immature embryo	13.30	33.30	63.30	60.00	73.30	73.30	23.30	40.00	46.67
Mean	41.65	54.15	72.50	40.00	65.65	70.15	24.99	45.00	50.00
	Full MS + 2,4,5-T 0.5 ppm			MS + 2,4,5-T 0.5 ppm			Quarter MS + 2,4,5-T 0.5 ppm		
Leaf	13.30	40.00	40.00	10.00	30.00	40.00	13.30	23.34	23.34
Male flower	13.30	26.70	36.70	10.00	10.00	30.00	23.30	43.33	43.33
Anther	23.30	50.00	60.00	21.10	66.60	66.60	20.00	36.67	36.67
Immature embryo	16.67	30.00	30.00	6.00	33.30	40.00	0.00	33.33	35.00
Mean	16.64	36.67	41.67	11.77	34.99	44.15	14.15	34.16	34.58

Data presented are mean of 3 replications.

Table 11. Effect of different levels of 2,4-D in combination with BAP for callus induction in *Musa* (AAB) 'Nendran'

Medium: full MS

Explant : Male flower

Treatment	Callusing %			Callus Index	Nature of calli	Days taken
	30 DAI	45 DAI	60 DAI			
2,4-D 0.5 ppm + BAP 0.5 ppm	22.0	46.6	46.6	65.24	Green friable calli	18-24
2,4-D 0.5 ppm + BAP 1.0 ppm	10.0	20.0	50.0	63.33	Green friable calli	18-24
2,4-D 0.5 ppm + BAP 2.0 ppm	22.4	43.3	43.3	57.73	Pale brown friable calli	18-24
2,4-D 0.5 ppm + BAP 4.0 ppm	26.6	31.3	46.6	59.03	Brownish calli	18-24
2,4-D 1.0 ppm + BAP 0.5 ppm	36.6	42.1	42.1	64.55	Brownish calli	18-24
2,4-D 1.0 ppm BAP 1.0 ppm	38.2	49.9	49.9	64.87	Brownish friable calli	18-24
2,4-D 1.0 ppm + BAP 2.0 ppm	14.3	34.3	33.3	42.19	Brownish friable calli	18-24
2,4-D 1.0 ppm + BAP 4.0 ppm	8.2	22.4	22.4	26.88	Brownish friable calli	18-24
2,4-D 2.0 ppm + BAP 0.5 ppm	33.3	42.2	42.2	63.3	Brownish friable Calli	18-24
2,4-D 2.0 ppm + BAP 1.0 ppm	42.3	50.0	64.2	106.99	Brownish friable calli	18-24
2,4-D 2.0 ppm + BAP 2.0 ppm	28.6	44.4	47.8	66.92	Brownish calli	18-24
2,4-D 2.0 ppm + BAP 4.0 ppm	14.4	31.3	31.3	42.78	Brownish calli	18-24

(64.20%) and callus index (106.99%) were observed with treatment combination of 2.0 ppm 2,4, D and 1.0 ppm BAP at 60 days after inoculation followed by 2,4-D 1.0 ppm and BAP 1.0 ppm having a callusing percentage of 49.9 and callus index of 64.87. Here the calli produced were brownish and friable in nature.

When 2,4,5-T was used with BAP for callus induction (Table 12) the response was very poor in all concentrations. The maximum callusing percentage of 46.6 at 60 days after inoculation was found in 2,4,5-T 0.25 ppm + BAP 1.0 ppm and the maximum callus index of 67.37 was recorded in the treatment combination 2,4,5-T 1.0 ppm + BAP 0.5 ppm. The calli produced were brownish, friable and having no regeneration potential. When the concentration of growth regulators increased, the calli produced were becoming compact in nature. The time taken for callus production was less for the 2,4,D and BAP combination, which produced callus within a period of 18-26 days while 2,4,5-T and BAP combinations took 30-62 days.

4.1.4 Comparative efficiency of 2,4-D and 2,4,5-T on callusing

The comparative efficiency of 2,4-D and 2,4-5-T on callus induction were tried using different concentrations of the two growth regulators (Table 13). 2,4-D at lower concentrations (0.1, 0.2, 0.3, 0.4 and 0.5 ppm) recorded the maximum callusing percentage and callus index compared to 2,4,5-T at the same concentration. 2,4-D at 0.5 ppm produced maximum calli and the callus index was also the highest followed by 2,4-D at 0.3 ppm with a callusing percentage of 96.0 (male flower) and 86.6 (immature embryo). The corresponding callus index were

Fig. 2. Response of male flowers and immature embryos in corrsponding concentrations of 2,4-D and 2,4,5-T

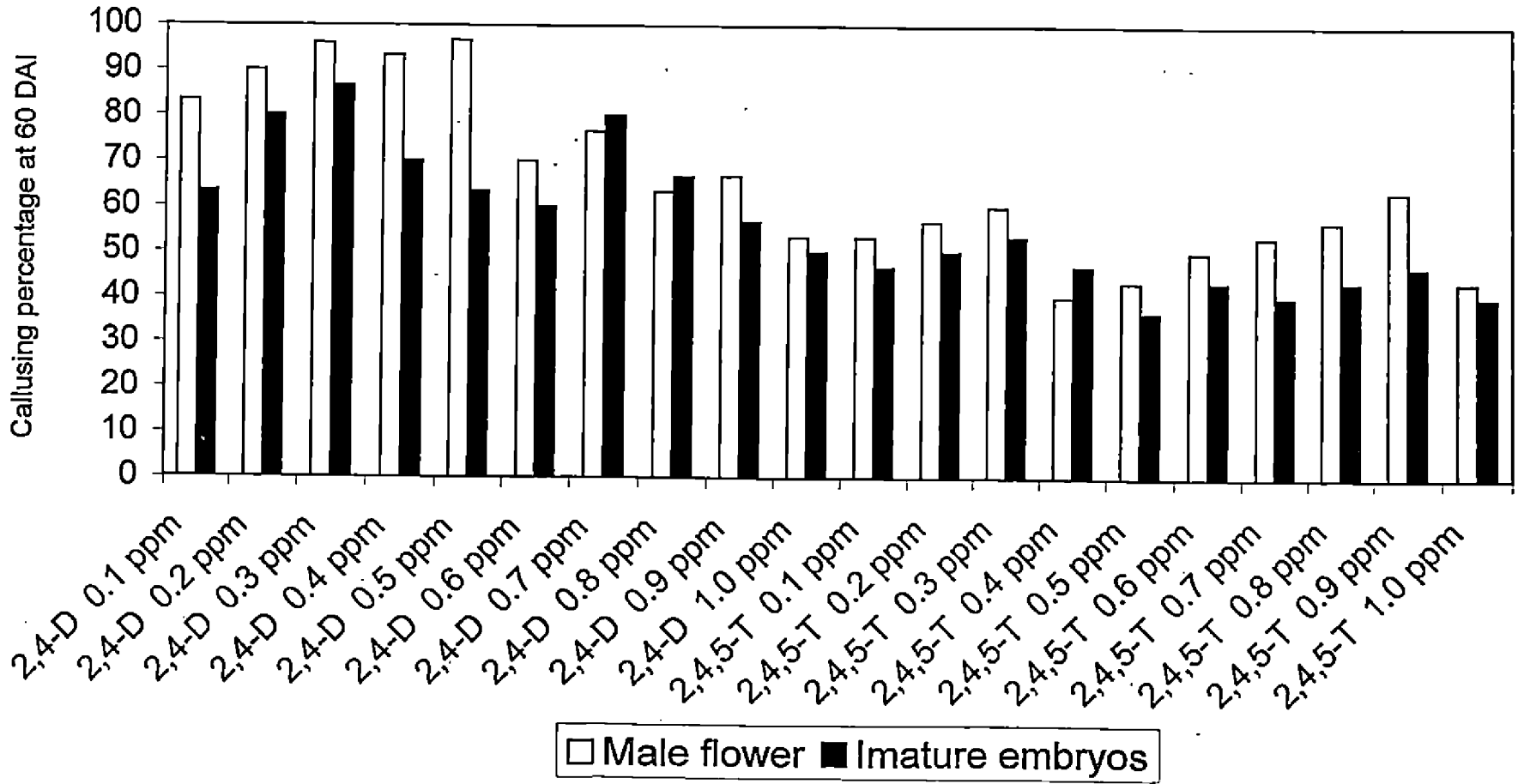


Table 12. Effect of different levels of 2,4,5-T in combination with BAP for callus induction in *Musa* (AAB) 'Nendran'

Medium: full MS
Explant : Male flower

Treatment	Callusing %			Callus index	Nature of callus	Days taken
	30 DAI	45 DAI	60 DAI			
2,4,5-T 0.25 ppm + BAP 0.5 ppm	0.00	33.30	33.30	49.95	Brown friable calli	35-62
2,4,5-T 0.5 ppm + BAP 0.5 ppm	0.00	16.67	40.00	64.00	Brown friable calli	35-62
2,4,5-T 1.0 ppm + BAP 0.5 ppm	0.00	20.00	43.00	67.37	Brown friable calli	35-62
2,4,5-T 2.0 ppm + BAP 0.5 ppm	0.00	23.30	33.30	51.06	Brown friable calli	35-62
2,4,5-T 0.25 ppm + BAP 1.0 ppm	0.00	26.60	46.60	59.02	Brown, friable, non regenerative calli	35-62
2,4,5-T 0.5 ppm + BAP 1.0 ppm	0.00	13.33	36.67	50.90	Brown friable, non regenerative calli	35-62
2,4,5-T 1.0 ppm + BAP 1.0 ppm	0.00	23.30	40.00	53.33	Brown, friable, bon regenerative calli	35-62
2,4,5-T 2.0 ppm + BAP 1.0 ppm	0.00	20.00	20.00	25.33	Brown, friable, non regenerative calli	35-62
2,4,5-T 0.25 ppm + BAP 2.5 ppm	0.00	16.60	22.90	27.48	Brown compact calli	35-62
2,4,5-T 0.05 ppm + BAP 2.0 ppm	0.00	10.00	26.60	34.58	Brown compact Calli	35-62
2,4,5-T 1.0 ppm + BAP 2.0 ppm	0.00	16.00	16.00	19.73	Brown compact calli	35-62
2,4,5-T 2.0 ppm + BAP 2.0 ppm	0.00	14.00	14.00	16.80	Brown compact calli	35-62

Table 13. Comparative study of efficiency of 2,4-D and 2,4,5-T on callus induction from male flowers and immature embryos

Treatments	Explant	Callusing % 60 DAI	Callus index	No. of days for callusing
2,4-D 0.1 ppm	Male flower	83.30	227.60	18-26
	Immature embryos	63.30	162.46	
2,4-D 0.2 ppm	Male flower	90.00	246.67	18-26
	Immature embryos	80.00	200.00	
2,4-D 0.3 ppm	Male flower	96.00	223.33	18-26
	Immature embryos	86.60	225.16	
2,4-D 0.4 ppm	Male flower	93.30	248.79	18-26
	Immature embryos	70.00	189.00	
2,4-D 0.5 ppm	Male flower	96.67	252.45	18-26
	Immature embryos	63.30	249.22	
2,4-D 0.6 ppm	Male flower	70.00	193.33	18-26
	Immature embryos	60.00	210.00	
2,4-D 0.7 ppm	Male	76.60	199.16	18-26
	Immature embryos	80.00	186.67	
2,4-D 0.8 ppm	Male flower	63.30	165.36	18-26
	Immature embryos	66.60	137.63	
2,4-D 0.9 ppm	Male flower	66.67	193.34	18-26
	Immature embryos	56.60	124.52	
2,4-D 1.0 ppm	Male flower	53.30	131.47	18-26
	Immature embryos	50.00	145.00	
2,4,5-T 0.1 ppm	Male flower	53.33	106.00	30-62
	Immature embryos	46.66	103.32	
2,4,5-T 0.2 ppm	Male flower	56.67	133.34	30-62
	Immature embryos	50.00	116.67	
2,4,5-T 0.3 ppm	Male flower	60.00	139.99	30-62
	Immature embryos	53.30	126.59	
2,4,5-T 0.4 ppm	Male flower	40.00	130.00	30-62
	Immature embryos	46.70	110.08	
2,4,5-T 0.5 ppm	Male flower	43.30	93.26	30-62
	Immature embryos	36.67	91.67	
2,4,5-T 0.6 ppm	Male flower	50.00	100.00	30-62
	Immature embryos	43.30	99.92	
2,4,5-T 0.7 ppm	Male flower	53.30	116.59	30-62
	Immature embryos	40.00	90.00	
2,4,5-T 0.8 ppm	Male flower	56.60	99.88	30-62
	Immature embryos	43.30	76.61	
2,4,5-T 0.9 ppm	Male flower	63.30	109.26	30-62
	Immature embryos	46.60	77.67	
2,4,5-T 1.0 ppm	Male flower	43.37	73.27	30-62
	Immature embryos	40.00	53.33	

Data presented are mean of 3 replications of each containing 10 treatments

223.33 and 225.16 respectively. The number of days taken for callus induction varied considerably for 2,4-D and 2,4,5-T. 2,4-D exhibited an early callusing while 2,4,5-T was late in callus induction process. Among the two explants tried, the male flower showed better callusing percentage and callus index compared to the immature embryo. More over, the callus produced by the male flower was having regeneration potential (Fig.2).

4.2 Effect of source of explant on callus induction

The source of explant and it's age has a major role in callus induction as revealed from the data recorded in Table 14. From the observations recorded it was found that most of the explants respond well for callus induction at younger stage except the male flower bases. The callusing property of male flower base explants collected from the outer rows of the male flowers was 96.67 percentage as against the 26.67 percentage recorded with flower bases collected from inner rows. The immature embryos collected from just opened hands showed the maximum callusing percentage than the fruits collected from one week old hands. In case of leaves also young cigar stage leaves produced better results than the mature leaves.

4.3 Effect of growth regulators in callus proliferation

4.3.1 Effect of 2,4-D on callus proliferation

The effect of 2,4-D on callus proliferation is presented in Table 15. Maximum callus proliferation was recorded with MS basal medium supplemented with 2,4-D 0.5 ppm followed by 2,4-D 0.3 ppm (100.0 and 99.0 percentage, respectively). The cultures under darkness for two weeks responded well for callus

Table 14. Effect of sources of explants on callus induction in Musa (AAB) Nendran

Medium: full MS + 2,4-D 0.5 ppm

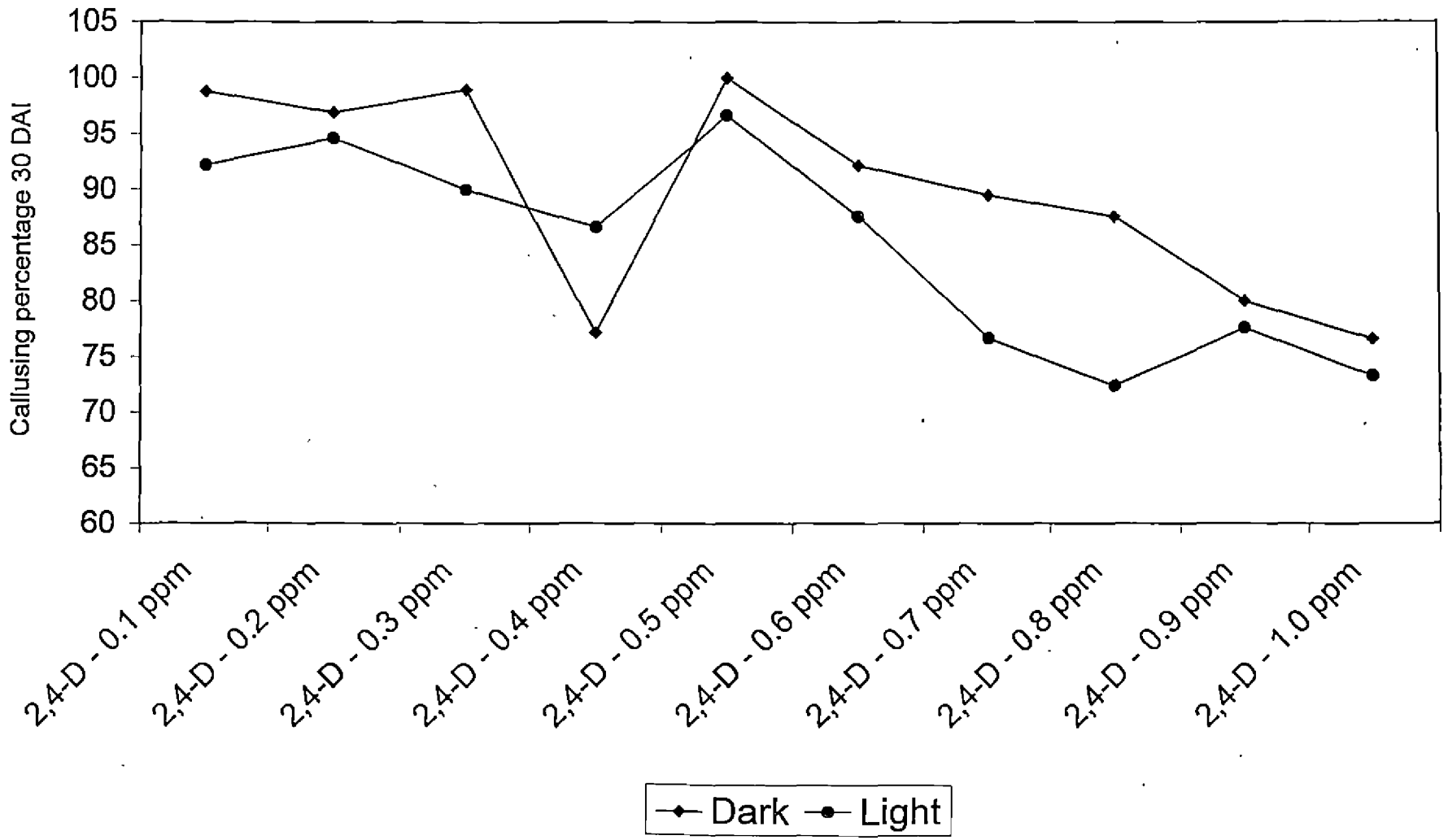
Explant	Source	Callusing (%)	Nature of callus
Leaf	Young stage/ cigar stage	53.3	Cream, compact calli from the joint between the leaf lamina and mid rib
	Mature leaves	16.67	Green compact calli which turned brown
In vitro Shoot	*In vitro* grown plants	36.87	The explants produced green compact callus
Leaf shoots	*In vitro* plant	0.00	No callus production was observed
	Field grown plant	0.00	No callus production
Root	*In vitro* plant	6.0	No callus production
	Immature stage/tip	10.0	Slight, cream calli produced from young roots
	Matured root	0.00	No callus production from mature roots
Anther	Immature stage	76.7	Profuse, white, friable calli
	Mature stage	20.0	No callus production from mature anthers
Meristem	Field grown plant	50.0	Brownish, compact calli which turned brown
Immature embryo	Emrbyos collected just after the opening of hands	63.3	White compact calli as the age of explant decreases callusing increase
	1 week after the opening	43.33	White, compact calli callus index low
Male flower	Flower bases collected from outer rows	96.67	White, friable calli
	Flower bases collected from inner rows	26.67	White friable calli. Callusing percentage was reduced compared to the basas collected from outer rows.

Table 15. Effect of 2,4-D in callus proliferation from male flower explants of Musa (AAB) Nendran

Media : full MS
Explant: Culture maintained in MS medium supplimented with 2,4-D 0.5 ppm

Treatment 2,4-D (ppm)	Dark		Light	
	% callus proliferation 30 DAI	Nature of callus	% callus proliferation 30 DAI	Nature of callus
0.10	98.80	White friable calli with regeneration potential	92.20	White friable calli with regeneration potential
0.20	96.90	White friable calli with regeneration potential	94.60	White friable calli with regeneration potential
0.30	99.00	White friable calli with hairy structure	90.00	White friable calli with hairy structure
0.40	77.20	„	86.67	„
0.50	100.0	„	96.66	„
0.60	92.20	White friable calli with regeneration potential	87.60	White friable calli
0.70	89.50	„	76.66	„
0.80	87.60	„	72.40	„
0.90	80.00	„	77.60	„
1.00	76.60	„	73.30	„

Fig. 3. Response of culture condition on callus proliferation



proliferation than the cultures kept under light condition. The treatment combination MS basal media + 2,4-D at 0.3 and 0.5 ppm maintained under dark condition for two months produced hairy structure from the callus (Fig.3).

4.3.2 Effect of combination of 2,4-D and NAA with BAP on callus proliferation

The effect of different combinations of 2,4-D and BAP on callus proliferation is presented in Table 16. The callus proliferation after 30 days of subculturing was recorded. The treatment combination 2,4-D at 2.0 ppm and BAP 1.0 ppm recorded the maximum callus proliferation rate at 30 days after sub culturing, having a value of 86.0, which is followed by 2,4-D at 2.0 ppm + BAP at 0.5 ppm having a value of 63.3 per cent. Under dark condition, the callus proliferation was maximum in all the treatment combinations except in 2,4-D at 1.0 ppm + BAP at 1.0 ppm and 2,4-D at 0.5 ppm + BAP at 4.0 ppm where the light conditions was more favourable.

Data on the response of different combinations of BAP and NAA on callus proliferation and the percentage of cultures induced embryogenic or non embryogenic calli are presented in Table 17. Maximum callus proliferation and embryogenic calli development were recorded in the treatment combination involving BAP at 2.0 ppm + NAA at 1.0 ppm, where 51.5 percentage of the cultures showed callus proliferation and percentage of cultures that induced embryogenic calli was 43.33. The immature embryos on sub culturing to this medium did not produced any embryogenic calli.

Table 16. Combination of 2,4-D and BAP in callus proliferation in *Musa* (AAB) 'Nendran'

Media: full MS
Explant: Calli maintained in MS basal media supplemented with 2,4-D 0.5 ppm

Treatment	Dark		Light	
	% callus proliferation 30 DAI	Nature of callus	% callus proliferation 30 DAI	Nature of callus
2,4-D 0.5 ppm + BAP 0.5 ppm	38.44	Pale brown friable calli	26.40	Pale brown friable calli
2,4-D 0.5 ppm + BAP 1.0 ppm	46.40	,,	32.60	,,
2,4-D 0.5 ppm + BAP 2.0 ppm	32.30	,,	23.30	,,
2,4-D 0.5 ppm + BAP 4.0 ppm	26.80	,,	43.30	,,
2,4-D 1.0 ppm + BAP 0.5 ppm	42.80	,,	30.00	,,
2,4-D 1.0 ppm + BAP 1.0 ppm	33.30	,,	46.60	,,
2,4-D 1.0 ppm + BAP 2.0 ppm	44.60	,,	28.22	,,
2,4-D 1.0 ppm + BAP 4.0 ppm	60.00	,,	46.60	,,
2,4-D 2.0 ppm + BAP 0.5 ppm	64.40	Brown calli	52.30	Brown calli
2,4-D 2.0 ppm + BAP 1.0 ppm	86.00	,,	76.60	,,
2,4-D 2.0 ppm + BAP 2.0 ppm	48.33	,,	24.60	,,
2,4-D 2.0 ppm + BAP 4.0 ppm	35.00	,,	20.00	,,

Table 17. Combined effect of BAP and NAA on callus proliferation from the male flowers of *Musa* (ABB) 'Nendran'

Media ; full MS
Explant : Callus maintained in MS media supplemented with 2,4-D 0.5 ppm

Treatment (ppm)	Explant	Light		Dark	
		% of cultures induced		% of cultures induced	
		Embryogenic callus	Non embryogenic callus	Embryogenic calli	Non embryogenic calli
BAP 1.0 + NAA 1.0	Male flower	10.00	90.00	0.00	100.00
BAP 2.0 + NAA 1.0	„	43.33	56.70	33.30	66.70
BAP 3.0 + NAA 1.0	„	26.67	73.33	13.30	86.70
BAP 4.0 + NAA 1.0	„	16.67	83.33	0.00	0.00
BAP 1.0 + NAA 2.0	„	0.00	0.00	0.00	0.00
BAP 2.0 + NAA 2.0	„	3.00	97.00	0.00	0.00
BAP 3.0 + NAA 2.0	„	0.00	0.00	0.00	0.00
BAP 4.0 + NAA 2.0	„	13.30	86.70	10.00	90.00
BAP 1.0 + NAA 3.0	„	0.00	0.00	0.00	0.00
BAP 2.0 + NAA 3.0	„	12.20	87.80	0.00	0.00
BAP 3.0 + NAA 3.0	„	0.00	0.00	0.00	0.00
BAP 4.0 + NAA 3.0	„	0.00	0.00	0.00	0.00

4.3.3 Role of NAA and kinetin on callus proliferation

Data on callus proliferation from the calli obtained from immature embryo cultured in MS media containing NAA and kinetin are presented in Table 18. The data shows that when the compact calli obtained from immature embryos were cultured in media containing NAA, no callus proliferation was noticed even after 60 days in culture. Kinetin, when used as growth regulator for callus proliferation, the result was very poor. At 45 days after inoculation, the calli exhibited slight callus proliferation where the calli produced were friable, non-embryogenic and brownish in colour.

4.4 Indirect organogenesis

4.4.1 Effect of different growth regulator combination on indirect organogenesis

4.4.1.1 Effect of auxins

Data on the response of 2,4-D on indirect organogenesis or rhizogenesis are recorded in Table 19. From the observation, it is clear that the response of the explant for indirect organogenesis or rhizogenesis was the highest in the concentration of 2,4-D at 0.5 ppm. The calli when cultured in MS medium containing 2,4-D at 0.5 or at 0.3 ppm recorded the maximum response to indirect organogenesis (100%) followed by 2,4-D at 0.4 and 0.1 ppm (99.9 and 99.0 per cent respectively). All the explants showed the development of hairy roots from the globular structures arising from the callus. The number of globular structures ranged from 10 to 20 per culture and the number of roots per culture was 3.0 to 6.6 on an average. The cultures kept under light condition produced more response to

Table 18. Effect of NAA and kinetin on callus proliferation from immature embryos of *Musa* (AAB) 'Nendran'

Media : full MS
Explant : Callus maintained in MS media supplemented with 2,4-D 0.5 ppm

Treatment	% callus proliferation			Callus index	Nature of callus
	30 DAI	45 DAI	60 DAI		
NAA					No callus proliferation was observed
0.1 ppm	0.00	0.00	0.00	Nil	
0.2 ppm	0.00	0.00	0.00	Nil	
0.3 ppm	0.00	0.00	0.00	Nil	
0.4 ppm	0.00	0.00	0.00	Nil	
0.5 ppm	0.00	0.00	0.00	Nil	
Kinetin					
0.1 ppm	0.00	20.00	33.30	46.62	Brownish friable non embryogenic calli
0.2 ppm	0.00	16.20	24.60	45.76	,,
0.3 ppm	0.00	22.30	30.00	46.00	,,
0.4 ppm	0.00	26.60	28.40	42.60	,,
0.5 ppm	0.00	26.60	23.30	34.17	,,

Table 19. Effect of 2,4-D in indirect organogenesis or rhizogenesis in *Musa* (AAB) 'Nendran'

Media : full MS
Explant: Calli maintained in MS medium supplemented with 2,4-D

Treatment 2,4-D (ppm)	Light			Dark		
	Nature of response	% culture showing response	Average No. of roots/ culture	Nature of response	% culture showing response	Average No. of roots/ culture
0.1	Profuse, white friable calli with globular structures and hairy roots	99.00	4.20	White friable calli, with globular structures and hairy roots	98.22	3.20
0.2	„	98.20	4.60	„	96.60	4.00
0.3	„	100.00	5.80	„	100.00	3.20
0.4	„	99.90	5.20	„	98.60	3.60
0.5	„	100.00	6.60	„	100.00	4.00
0.6	White friable calli with globular structure and hairy roots	96.60	5.40	„	99.90	3.40
0.7	„	88.70	3.80	„	93.30	3.00
0.8	White friable calli with regenerative potential and hairy roots	92.20	4.00	„	87.70	3.40
0.9	„	86.60	4.20	„	92.20	2.00
1.0	„	88.60	3.00	„	85.50	3.00
1.5	White friable calli without hairy roots	80.00	0.00	White friable calli without hairy roots	76.60	0.00
2.0	„	72.20	0.00	„	64.40	0.00

Data presented are average of 5 cultures

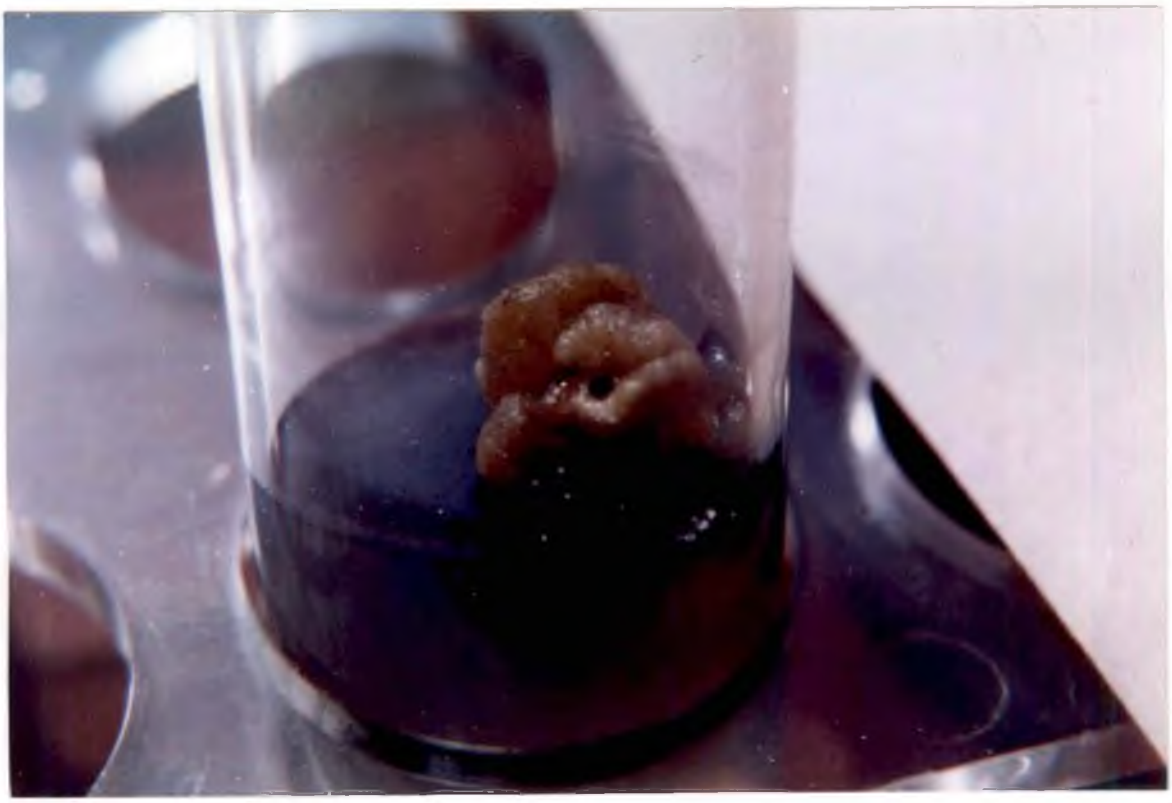


Table 21. Effect of GA_3 on indirect organogenesis in *Musa* (AAB) 'Nendran'

Media : full MS
Explant: Calli maintained in full MS medium supplemented with 2,4-D 0.5 ppm

Treatment (ppm)	Light			Dark		
	Nature of response	% cultures showing response	No. of roots/ culture	% cultures showing response	No. of roots/ culture	Nature of response
GA_3 0.1	Hairy root formation	68.40	3.50	65.30	3.20	Hairy root formation
GA_3 0.5	,,	52.60	3.20	43.30	3.00	,,
GA_3 1.0	,,	43.30	0.00	52.20	0.00	,,
GA_3 1.5	,,	40.00	0.00	33.30	0.00	,,
GA_3 0.1 + BAP 4.0	,,	72.20	3.50	63.30	3.50	,,
GA_3 0.5 + BAP 4.0	,,	86.30	3.80	68.10	3.50	,,
GA_3 1.0 + BAP 4.0	,,	63.30	3.00	52.20	3.00	,,
GA_3 1.5 + BAP 4.0	Brownish calli with globular structure	62.60	0.00	46.67	0.00	Brownish calli with globular structure
GA_3 2.0 + BAP 4.0	,,	52.20	0.00	23.30	0.00	,,
GA_3 4.0 + BAP 4.0	,,	50.00	0.00	20.00	0.00	,,
GA_3 6.0 + BAP 4.0	,,	23.00	0.00	26.60	0.00	,,

4.4.1.4 Effect of kinetin

When kinetin alone was used with MS basal medium, the response was inhibitory. The cultures became dark and the globular structures initiated before sub culturing turned brown.

4.5 Somatic embryogenesis

4.5.1 Induction of somatic embryos

4.5.1.1 Effect of different basal media and culture conditions on somatic embryogenesis

The different basal media and culture conditions tried for the induction of somatic embryos are presented in Table 22. For the induction of somatic embryos, full MS medium was tried. The MS medium having 2,4-D at 0.5 ppm and 1.0 ppm produced more embryogenic calli (100%). Among the different combinations of BAP and NAA tried, the treatment combination involving BAP at 2.0 ppm and NAA at 1.0 ppm induced the highest per cent of cultures with embryogenic calli (43.33%). In most other treatment combinations, the explants produced more non-embryogenic calli. The light condition favoured the formation of more amount of embryogenic calli.

The effect of different growth regulators on the formulation of embryogenic calli showed that 2,4-D at 0.5 ppm and 1.0 ppm concentration induced the formation of globular structures on the callus surface. Among these two concentrations of 2,4-D, the globular structure formation was higher at 0.5 ppm. All the other treatments tried for somatic embryo formation i.e. full MS media having BAP + NAA, kinetin + NAA and full MS medium having BAP, also did not produce any somatic embryos.

Table 22. Effect of different basal media, growth regulator and culture conditions on induction of somatic embryos in *Musa* (AAB) 'Nendran'

Explant : Calli maintained in MS medium supplemented with 2,4-D 0.5 ppm

Treatment (ppm)	Light		Dark	
	% of cultures showing somatic embryos	% of cultures showing embryogenic calli	% of cultures showing somatic embryos	% of cultures showing embryogenic calli
MS (basal media)	0.00	100.00	0.00	100.00
MS 2,4-D 0.1	0.00	100.00	0.00	96.60
MS 2,4-D 0.5	0.00	90.00	0.00	100.00
MS 2,4-D 1.0	0.00	97.00	0.00	92.30
MS 2,4-D 1.5	0.00	100.00	0.00	96.60
MS + BAP 1.0 + NAA 1.0	0.00	10.00	0.00	0.00
MS + BAP 2.0 + NAA 1.0	0.00	43.33	0.00	33.30
MS + BAP3.0 + NAA 1.0	0.00	26.67	0.00	13.30
MS + BAP 4.0 + NAA 1.0	0.00	16.67	0.00	0.00
MS + BAP 1.0 + NAA 2.0	0.00	0.00	0.00	0.00
MS + BAP 2.0 + NAA 2.0	0.00	3.00	0.00	0.00
MS + BAP 3.0 + NAA 2.0	0.00	0.00	0.00	0.00
MS + BAP 4.0 + NAA 2.0	0.00	13.30	0.00	10.00
MS + BAP 1.0 + NAA 3.0	0.00	0.00	0.00	0.00
MS + BAP 2.0 + NAA 3.0	0.00	12.20	0.00	0.00
MS + BAP 3.0 + NAA 3.0	0.00	0.00	0.00	0.00
MS + BAP 4.0 + NAA 3.0	0.00	0.00	0.00	0.00
Half MS + 2,4-D 0.1	0.00	96.60	0.00	90.00
Half MS + 2,4-D 0.5	0.00	100.00	0.00	99.90
Half MS + 2,4-D 1.0	0.00	90.00	0.00	83.30
Half MS + 2,4-D 1.5	0.00	84.30	0.00	80.00
Half MS + BAP 1.0 + NAA 1.0	0.00	13.30	0.00	3.00
Half MS + BAP 2.0 + NAA 1.0	0.00	40.00	0.00	26.60
Half MS + BAP 3.0 + NAA 1.0	0.00	16.60	0.00	10.00
Half MS + BAP 4.0 + NAA 1.0	0.00	0.00	0.00	0.00
Half MS + BAP 1.0 + NAA 2.0	0.00	0.00	0.00	0.00
Half MS + BAP 2.0 + NAA 2.0	0.00	6.00	0.00	3.00
Half MS + BAP 3.0 + NAA 2.0	0.00	0.00	0.00	0.00
Half MS + BAP 4.0 + NAA 2.0	0.00	0.00	0.00	0.00
Half MS + BAP 1.0 + NAA 3.0	0.00	6.00	0.00	0.00
Half MS + BAP 2.0 + NAA 3.0	0.00	13.30	0.00	6.00
Half MS + BAP 3.0 + NAA 3.0	0.00	0.00	0.00	0.00
Half MS + BAP 4.0 + NAA 3.0	0.00	0.00	0.00	0.00
MS + Kinetin 0.5 NAA 0.5	0.00	46.60	0.00	0.00
MS + Kinetin 1 NAA 0.5	0.00	33.30	0.00	0.00
MS + Kinetin 2 NAA 0.5	0.00	0.00	0.00	0.00
Ms + Kinetin 0.5 NAA 1	0.00	26.60	0.00	0.00
MS + Kinetin 1 NAA 1	0.00	10.00	0.00	0.00
MS + Kinetin 2 NAA 1	0.00	0.00	0.00	0.00

Discussion

DISCUSSION

Somatic embryogenesis is a useful technology for the large scale production of plant materials within a short period of time. In bananas, this helps to produce good quality planting material in large number from a selected single mother plant. An appropriate technology for the production of somatic embryos should therefore be standardised in this species. For this, an appropriate protocol including the most suitable media and explant should be standardised. Results of the experiments carried out to achieve this objective are discussed below.

5.1 Callus induction

5.1.1 Standardisation of suitable media and explant

The standardisation of a suitable media is the first step in any tissue culture programme. The media requirement in the different routes i.e., anther culture, protoplast culture, embryoculture, somatic embryogenesis etc. also differ. The most widely used medium for plant tissue culture work is the MS medium (Murashige and Skoog, 1962).

In the present study, MS medium varying with respect to concentration of macro and micro nutrients were tried for callus induction and for its further proliferation.

Souza and Goncalves (1996) obtained shoot proliferation and root development in *Musa* cv. vanicao when cultured in Murashige and Skoog medium. The effectiveness of MS medium on callus induction and callus proliferation were recorded using various explants like meristematic shoot

(Panis *et al.*, 1992; Reddy, 1996), male flower primordia (Ma *et al.*, 1989; Gomez-Kosky *et al.*, 1994; Navarro *et al.*, 1997; Ganapathi *et al.*, 1999), rhizome bits (Novak *et al.*, 1989; Domingues *et al.*, 1996; Lee *et al.*, 1997), immature embryos (Escalent and Teisson, 1988b; Pancholi *et al.*, 1995; Navarro *et al.*, 1997), leaf primordia and leaf basis (Novak *et al.*, 1989; Shchukin *et al.*, 1997), pseudostem (Domingues *et al.*, 1996) and leaf explants (Jarret *et al.*, 1985).

Among the different concentrations of MS tried, the full strength MS media produced better results than half or quarter MS media. Among the different explants tried namely immature embryo, male flower, leaf, root tip, leaf sheath, anther, *in vitro* shoot and root tips and meristem, the immature embryo and male flower produced profuse calli on MS medium while the leaf produced only slight callusing. Anthers were much better in their callusing property but the calli produced were non embryogenic in nature. The immature embryo produced better results in half MS medium.

5.1.2 Effect of different growth regulators on callus induction

5.1.2.1 Effect of auxin

Different explants from the Nendran variety of banana were cultured on different strengths of MS medium with various levels of 2,4-D, NAA and 2,4,5-T. The best growth regulator for callus induction was 2,4-D compared to the other two auxins. The callus inducing property of NAA was very negligible. Maximum callus index and callusing percentage were recorded with 2,4-D at 0.5 ppm.

A number of workers have reported the use of 2,4-D for callus induction. Litz (1985) reported the induction of embryogenic calli from explants of herbaceous arborescent plants of papaya, banana and plantain by culturing them in MS medium containing 1.0 to 2.0 mg^{-1} 2,4-D. Callus can be initiated from floral or inflorescence tissue in the presence of 2,4-D (Bakry and Rossignol, 1985).

Babu *et al.* (1992) obtained callus growth and proliferation at 9.0 to 22.6 µM of 2,4-D in ginger. Callus inducing property of 2,4-D when used at 0.5, 1.0 or 2.0 mg^{-1} were also reported by Cronauer and Krikorian (1988).

The highest callusing percentage was recorded at 0.25 ppm 2,4,5-T. Other concentration were not that much effective in callus production. Time taken for callusing was longer for 2,4,5-T than 2,4-D.

When NAA was used there was no callusing in any of the explant except in leaf and *in vitro* shoot, where a slight callusing was observed.

5.1.2.2 Effect of growth regulator combination

Among the different growth regulator combinations attempted, the highest callusing percentage (64.2) and callus index (106.99) were observed with 2.0 ppm 2,4-D and 1.0 ppm BAP combination. The calli produced were brownish, friable and hairy with regeneration potential. Higher concentrations of BAP with 2,4-D also initiated callus growth but the calli produced became dark. When 2,4,5-T was used along with BAP the callus induction rate was very poor.

Skoog and Miller (1957) have reported that the levels of plant growth regulating substances in the culture medium particularly high auxin and low cytokinins, often lead to callus formation.

Dhed'a (1992) could obtain embryogenic cell suspension cultures from shoot tip explants of five varieties of banana on MS medium supplemented with 5.0 μM 2,4-D and 1.0 to 10.0 μM BA or zeatin. Cronauer and Krikorian (1984a) have reported the production of proembryos in a media containing basal MS salts and 2,4-D or 2,4,5-T along with BAP.

The callus inducing property of 2,4-D was nullified with the addition of kinetin. The explant turned brown within 60 days of inoculation.

5.1.2.3 Comparative efficiency of 2,4-D and 2,4,5-T on callus induction

From this experiment, it was found that 2,4-D was much better in terms of percentage callusing as well as earliness in callusing than 2,4,5-T. When 2,4-D was used in the culture medium, the explants produced an early callusing, compared to 2,4,5-T which was much slower in its callus inducing property. 2,4,5-T took 33-35 days while 2,4-D produced callus within 18-26 day.

5.2 Source of explants for callus induction

The best results were obtained with explants like male flower, immature embryo and anther. The source as well as the age of explants play a major role in callus induction. There are many reports on the use of immature parts of *Musa* spp. for callus induction. Many scientists have worked on this subject and they used immature embryo or young male flower for callus induction Navarro *et al.* (1997) have reported the use of immature fruits of the diploid species *Musa acuminata* (AA) ssp. *malaccensis,* harvested 60 days after fertilization. They have also reported that in Grand Naine (AAA) male inflorescence (distal end) were ideal for callus induction.

Somatic embryogenesis has been reported from leaf sheath or rhizome explant from *in vitro* plants, immature zygotic embryo and male flower bud cultures (Marroquin *et al.*, 1993). The male inflorescence collected from field grown plants were surface cleaned with absolute alcohol. Under a sterile laminar air flow cabinet, the outer enveloping bracts were removed and the inner part (2-3 cm in length) containing male flower primordia were cultured on Murashige and Skoog medium (Ganapathi *et al.*, 1999).

Eventhough callus was produced by immature embryo and anther, the calli were not having any embryogenic potential. The highest callusing percentage was observed with flower base collected from inner rows.

5.3 Effect of growth regulators in callus proliferation

5.3.1 Effect of auxin

Novaille and Petiard (1988) reported that, in an experiment conducted to produce somatic embryos of plants like celery, lettuce, tomato, potato or forest trees or fruit trees such as apple and banana, callus developed *in vitro* in a medium containing auxin.

During the present investigation it was found that the auxin media was suitable for callus proliferation. And the maximum callus proliferation was recorded with 2,4-D at a concentration of 0.5 ppm. Dark incubation induced better callus proliferation than light incubation.

NAA has no effect on callus proliferation. When the calli were cultured in MS medium containing NAA, no callus proliferation was observed even after 60 days.

5.3.2 Effect of kinetin

In banana, kinetin did not express any enhancing effect on callus induction or callus proliferation. When calli were subcultured into the MS media supplemented with kinetin, the cultures became dark and did not produce any callus proliferation. Thus it was found that kinetin is not suitable for callus induction or callus proliferation from the explants.

5.3.3 Effect of combination of auxin and cytokinin

Cronauer and Krikorian (1984b) have reported that shoot clusters of the plantains Saba and Pelipita grown in liquid medium composed of basal MS salts with 2,4-D or 2,4,5-T and BAP produced small pro-embryonic masses.

In the present study, it was found that among the different combinations of 2,4-D with BAP, 2,4-D at 2.0 ppm and BAP at 1.0 ppm recorded the maximum rate of callus proliferation, which is followed by 2,4-D at 2.0 ppm with BAP at 0.5 ppm.

Among the different combination of BAP and NAA tried, the maximum callus proliferation was recorded with the treatment combination involving BAP and NAA at 2.0 and 1.0 ppm respectively.

5.4 Indirect organogenesis

5.4.1 Effect of auxins.

Nair and Seo (1993) have reported callus regeneration from *Allium senescens* var. minor in MS medium supplemented with lower levels of 2,4-D (4.5 µM). Many scientists have reported the development of somatic embryos from different explants of banana by culturing in MS media supplemented with, 2,4-D, dicamba etc.

From this study it was found that 2,4-D when used alone, can induce organogenesis in *Musa* (AAB) Nendran. When 2,4-D was used at lower concentrations i.e., 0.5 ppm and 1.0 ppm, it induced root formation from the calli obtained from male flower explants.

5.4.2 Combined effect of auxin and cytokinin

Cronauer and Krikorian (1984a) have reported the development of long roots with well developed root hairs, when the explants were cultured in liquid medium composed of basal MS salts and 2,4-D or 2,4,5-T along with BAP. Bakry and Rossignol (1985) have reported that callus from fragments of inflorescence apex could be cultured in medium lacking 2,4-D or 2,4,5-T without blackening or necrosis and were capable of organogenesis after several subcultures in a media containing Murashige and Skoog basal salts with 2 mg^{-1} each of IAA and BA.

But the present study reveals that when 2,4-D was used along with BAP, the result was much better. 2,4-D at 2.0 ppm and BAP 1.0 ppm produced pronounced results among the different combinations tried, followed by 2,4-D at 2.0 ppm with BAP at 0.5 ppm.

5.4.3 Effect of GA_3

It was found that GA_3 reduces somatic embryogenesis in banana but the germination of preformed embryo of several different crop species can be stimulated by the incorporation of GA_3. Joseph (1997) have reported that, GA_3 did not express any enhancing or inhibiting effect on indirect organogenesis even when used in combination with BA. In *Kaempferia galanga,* the percentage and average number of shoots produced were less when GA_3 was used with BA than that with

BA alone. There was a negative effect on culture establishment when GA_3 was used for culturing shoot tip, eye bud or floral apex explants of banana (Bhaskar *et al.,* 1993). Gibberellin along with benzyladenine enhanced somatic embryo germination (Ma *et al.,* 1989).

This study on somatic embryogenesis reveals that GA_3 did not produce any promoting or inhibitory effect on indirect organogenesis, when it was used alone or in combination with BAP.

5.4.4 Effect of kinetin

Many studies on somatic embryogenesis reveals that for the maturation of somatic embryos, kinetin was used. Miller *et al.* (1956) have reported that kinetin stimulated cell division in cells that otherwise might have become multinuclear. The addition of kinetin increased the callus water potential by increasing the turgor potential and thereby reducing the water uptake from the surrounding medium.

In the present study, when the calli having roots and globular structures transferred to the MS medium having kinetin, the culture became dark within one week.

5.5 Somatic embryogenesis

Navarro *et al.* (1997) have reported that in banana somatic embryos formed in Murashige and Skoog media containing 2,4-D. According to Lee *et al.* (1997) in banana somatic embryos developed in half strength Murashige and Skoog medium with 3 μM 2,4-D, under dark condition.

The different studies carried out using immature male flowers of banana and plantain to optimize the culture medium including gelling agent and 2,4-D levels it was found that after three months in culture, the flower produced yellow coloured callus. After 4-6 months without subculturing a translucent callus formed over the yellow callus and many somatic embryos were observed on the callus surface. Here incubation under dark gave an increased number of embryogenic calli compared to incubation under natural or artificial light (Del-Sol *et al.*, 1995).

Somatic embryos were developed in triticale from immature inflorescence using 2,4-D (4.0 mg^{-1}). At this concentration of 2,4-D, banana shoot buds also produced somatic embryos (Reddy, 1996).

Ma *et al.* (1989) have reported that somatic embryos developed from the inflorescence tips of one diploid and two triploid cultivars of banana, when grown on semi-solid Murashige and Skoog culture medium containing 2,4-D alone or in combination with three other auxins. In another report, somatic embryos developed from near the shoot apical meristem of the zygotic embryo, in presence of relatively high levels of 2,4-D.

For the germination of somatic embryos, it requires a medium containing more amount of cytokinins. In general, auxins favour root formation and cytokinins, shoot formation. Since the somatic embryos have both root and shoot primordia, no separate rooting media is required for root formation.

In the present study it was found that globular structures developed in full strength Murashige and Skoog culture medium containing 2,4-D at 0.5 ppm. Male flower was the most suitable explant. Globular structures developed on the surface of the callus after about two months. On subculturing these globular structures to maturation media containing kinetin or BAP, they became dark within 60 days.

Bakry and Rossignol (1985) found that, when the floral explants were cultured in presence of 2,4-D, there was organogenesis from the callus. When the leaf explants were cultured in presence of 2,4-D or 2,4,5-T, only root formation was observed.

Among the different experiments carried out on somatic embryogenesis, there are reports on the development of roots from the somatic embryo without any shoot formation. Cronauer and Krikorian (1984a) have reported the development of roots with well developed root hairs. However no shoot formation was observed. Jarret *et al.* (1985) have observed the development of roots from leaf base explants. They were able to obtain enlarged and elongated globular masses from the explant when cultured in MS medium, which closely resembled the somatic embryos. Shoot formation was however never observed.

Summary

SUMMARY

In the present study, in order to standardise a suitable explant and growth regular for somatic embryogenesis, different explants of the banana variety, Nendran were tried with three concentrations of MS medium having different combinations of growth regulators. The explants used for the study, includes, leaf, leaf sheath, root, *in vitro* root and shoot tips, immature embryo, meristem and anther. The results of the studies on "Somatic embryogenesis in *Musa* (AAB) 'Nendran'", are summarised in this chapter.

1. For callus induction, half MS medium produced better results for leaf segment and male flowers. For immature embryo and anther, the full MS medium showed better results. The effects of full MS media was superior followed by half MS media on callus induction where the quarter MS media exhibited least value.
2. Among the different explants used, the male flower and immature embryo produced better results. The male flower was more suitable for somatic embryogenesis because they produced more embryogenic calli having more embryogenic potential. Eventhough the immature embryos produced prolific calli, the embryogenic potential was lower. Other explants did not produce any embryogenic calli.
3. For callus induction, auxin was the most suitable growth regulator, especially when used at a lower concentration i.e., at 0.5 ppm, 2,4-D induced better callusing than any other growth regulator at any concentration.

4. When, 2,4,5-T was used for callus induction the effect was slow. Eventhough callusing was observed in certain explants, the time taken was much longer when compared to 2,4-D. Here also the lower concentrations i.e., 0.5 ppm and less gave better results compared to higher concentrations of 2,4,5-T.
5. When NAA was used, callusing was not observed from any explant.
6. The growth regulator combination involving 2,4-D at 2.0 ppm and BAP at 1.0 ppm also produced callus from immature embryos and male flowers. Here the response was not better than the response produced by 2,4-D alone. The callus inducing property of other combinations of 2,4-D and BAP were not notable.
7. The 2,4,5-T and BAP combination was effective for callus induction but the time taken was longer (30 to 62 days). 2,4,5-T in general will take more time for callus induction.
8. When the callus inducing property of 2,4-D and 2,4,5-T were compared, it was found that 2,4-D was better for callus induction, than 2,4,5-T because of the higher callus index and earliness in callusing when the male flowers were cultured in media containing 2,4-D (0.5 ppm) it produced more embryogenic calli and somatic embryos.
9. Mostly the young stage of any explant is more suitable for callus induction. Except in male flower where the mature stage produced better results, all other explants exhibited better response at their younger stage.

10. For callus proliferation, i.e., large scale multiplication of callus, and for the production of embryogenic calli, different growth regulators were tried alone or in combination. It was found that the growth regulator 2,4-D was suitable for callus proliferation also. The combinations of 2,4-D with BAP were also favourable for callus proliferation.
11. When BAP was used in combination with NAA the effect was negative. No embryogenic calli were produced and only non-embryogenic calli were produced.
12. No callus proliferation was observed when the calli obtained from imature embryos were sub cultured to a medium containing NAA.
13. Kinetin when used for subculturing, the effect was negative. The calli turned brown within two weeks.
14. When the calli were sub cultured in a media containing 2,4-D at lower concentrations i.e., 0.3, 0.4 and 0.5 ppm,it produced roots with a number of root hairs. The roots originated from small globular structures, which were present in large number per culture. On further sub culturing the roots increased in length.
15. When 2,4-D was used in combination with BAP, large number of roots were formed. The combination involving 2,4-D at 2.0 ppm and BAP at 1.0 ppm produced better results.
16. When GA_3 was used for subculturing the calli, it did not produce any inhibiting effect on rhizogenesis. The callus produced globular structures and from these structures roots were produced.

17. When GA_3 was used in combination with BAP also there was no inhibiting effect on rhizogenesis. However, at higher concentrations of GA_3 the explants turned brown and the media also became discoloured.
18. When kinetin was incorporated in the medium for subculturing the calli, it showed an inhibitory effect on rhizogenesis. Here, the globular structures, which were produced before subculturing to the kinetin medium, became brown within 30 days.
19. Somatic embryo formation was not observed from any of the treatment combinations tried.

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SOMATIC EMBRYOGENESIS IN *Musa* (AAB) 'NENDRAN'

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ABSTRACT OF THE THESIS

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ABSTRACT

A study was taken up in the Department of Pomology and Floriculture, College of Horticulture during 1998-2000 to standardise a suitable explant as well as growth regulator for somatic embryogenesis in *Musa* (AAB) 'Nendran'. The ecotype Manjeri Nendran was used for study.

The different explants tried includes meristem, male flower, immature embryo, leaf sheath, anther, root, in vitro derived root and shoot tips.

The medium tried were full MS, half MS and quarter MS having different concentration of various growth regulators like 2,4-D, 2,4,5-T, NAA, Kinetin, GA_3, BAP etc.

From this experiment it was found that regarding the concentration of MS medium the full MS medium was the best for male flowers, while the half MS medium was more suitable for anther and immature embryo.

Among the different explants tried for callus induction, best results were obtained with anther, male flower and immature embryo. Eventhough the anther and immature embryo produced profuse calli, they were not having any embryogenic potential, however the calli produced by the male flowers produced while globular structures which in turn gave rise to hairy roots.

Regarding the different growth regulators tried for callus induction, best results were observed with 2,4-D. 2,4-D when used at 0.5 ppm level induced better callusing in many of the explants. The higher concentrations of 2,4-D i.e. 1.0 ppm or 2.0 ppm were not that much effective for callus induction.

For callus proliferation also 2,4-D was the best growth regulator, 2,4-D at 0.5 ppm or less produced profuse callusing. This is followed by the treatment combination involving 2,4-D 2 ppm + BAP 1 ppm.

Rhizogenesis was observed when the calli were subcultured to a medium containing 2,4-D, or GA_3, or a combination of 2,4-D + BAP or GA_3 + BAP.

Regarding the culture conditions suitable for callus induction, callus proliferation and organogenesis, the light condition favoured callus induction and organogenesis while the dark condition enhanced callus proliferation.