INDUCTION OF GENETIC VARIABILITY IN *Musa* Sp. Var. NENDRAN BY *in vitro* METHODS

By

MINI BALACHANDRAN

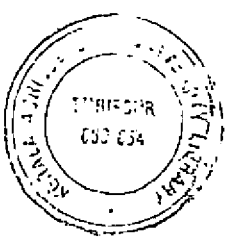
THESIS

Submitted in partial fulfilment of the
requirement for the degree

Master of Science in Horticulture

Faculty of Agriculture
Kerala Agricultural University

1993



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I hereby declare that the thesis entitled 'Induction of genetic variability in *Musa* sp. var. Nendran by *in vitro* methods' is a bonafide record of work done by me during the course of research work and the 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.

College of Horticulture
Vellanikkara
/ -12-1993

MINI BALACHANDRAN

DR.P.K.VALSALAKUMARI
Associate Professor

College of Horticulture
Vellanikkara

CERTIFICATE

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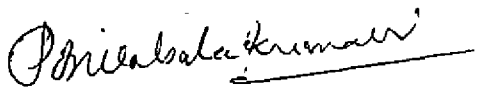

Dr.P.K.Valsalakumari
Chairperson
Advisory Committee

CERTIFICATE

We the undersigned, members of the Advisory Committee of **Miss.Mini Balachandran,** a candidate for the degree of Master of Science in Horticulture with major in Pomology and Floriculture, agree that the thesis entitled 'Induction of genetic variation in Musa sp. var. Nendran by in vitro methods' may be submitted by Miss.Mini Balachandran, in partial fulfilment of the requirements for the degree.

Chairperson **Dr.P.K. Valsalakumari**
Associate Professor
Dept. of Pomology and
Floriculture

Members **Dr.P.K. Rajeevan**
Professor & Head i/c
Dept. of Pomology and
Floriculture

Dr.P.A. Wahid
Professor
Radio Tracer Laboratory
College of Horticulture

Dr.R. Keshavachandran
Assistant Professor
Dept. of Plantation Crops
and Spices

ACKNOWLEDGEMENT

I consider it a privilege to express my heartfelt thanks and indebtedness to Dr.P.K.Valsalakumari, Associate Professor, Department of Pomology and Floriculture and Chairperson of my advisory committee for her keen interest, sustained guidance, constant encouragement and patience throughout the course of this investigation and preparation of the manuscript.

I express my profuse and sincere gratitude to Dr.P.K.Rajeevan, Professor and Head i/c, Department of Pomology and Floriculture for his everhelping nature in the completion of my research programme. And also I am highly grateful to him for the help rendered in the photographic works.

I am very much grateful to Dr.P.A.Wahid, Professor, Radio Tracer Laboratory, for his co-operation and help during the course of study.

I wish to express my deep sense of gratitude to Dr.R.Kesha-vachandran, Assistant Professor, presently in the Department of Plantation Crops and Spices for his constructive criticism and valuable advice during the course of investigation as well as in the preparation of the thesis.

I owe a lot to Dr.C.K.Geetha, Assistant Professor, Department of Pomology and Floriculture, for her keen interest in the study and immense help rendered during the course of research work. I take this opportunity for expressing my profound sense of gratitude to her.

I am also thankful to all the members of the staff of the Department of Pomology and Floriculture for the co-operation and assistance at various stages of this investigation.

I am gratefully obliged to Dr.Radhakrishnan, Associate Professor, Banana Research Station, Kannara and all the staff members for providing the explant materials and sincere help rendered throughout the course of investigation. I am very much grateful to them.

I am also grateful to Sri.V.K.Raju, Associate Professor and Dr.S.Nirmaladevi, Assistant Professor for providing necessary help at various stages.

I thank Dr.C.C.Abraham, Associate Dean, College of Horticulture for the facilities provided for undergoing the M.Sc.(Hort) programme.

I am also grateful to Sri.V.Kunju and Sri.C.B.Sugathan for the necessary assistance. My sincere thanks are also due to Sri.Raman, Smt.Kowjumma, Smt.Pathumma and all the others for their sincere co-operation and assistance rendered to me during the course of the laboratory work.

I am very much thankful to Sri.Joy for the neat typing and prompt service.

My sincere thanks go to Sri.Santhosh Kumar,A.V. for his timely help during the analysis of data and in preparing the graphs.

The whole hearted co-operation, valuable advice and assistance rendered by my class mates had been of immence help to me in overcoming the many difficult situations faced by me and finishing my work in time. Let me express my heartfelt gratitude to them. Also my sincere thanks to all my friends who have assisted me throughout my work.

I wish to acknowledge Kerala Agricultural University for awarding the Junior Research Fellowship.

I am forever beholden to my parents and sister who kept encouraging me at all critical junctures. Also I am very much indebted to my uncle, for his constant encouragement and help during the period of study.

Last, but not least, I pay Homage to late Sri.M.K.Mammen, former Professor and Head of the Department of Pomology and Floriculture, who suggested the topic for my study and I believe whose blessings were always with me in undertaking this endeavour successfully.

Above all, I bow my head before God Almighty for enabling me to complete this small venture of mine successfully.

MINI BALACHANDRAN

CONTENTS

ABBREVIATIONS

cm	-	centimeter
mm	-	millimeter
um	-	micrometer(s)
h	-	hour(s)
min	-	minute(s)
ml	-	millilitre
mg/l	-	milligram per litre
pH	-	hydrogen ion concentration
ppm	-	parts per million
C	-	degree celsius
AC	-	Activated charcoal
BA	-	Benzyladenine
2,4-D	-	2,4-dichlorophenoxy acetic acid
2,4,5-T	-	2,4,5-trichlorophenoxy acetic acid
KIN	-	Kinetin
EDTA	-	Ethylene diamine tetra acetate
LS	-	Linsmair and Skoog (1965)
MS	-	Murashige and Skoog's medium (1962)
SH	-	Schenk and Hildebrandt (1972)

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Introduction

INTRODUCTION

Banana (Musa sp.) is one of the most important tropical fruits of the world. India ranks second in area under this crop (3.39 lakh ha), with an annual production of 61.76 lakh tonnes. Kerala is one of the major banana growing tracts in India, occupying an area of 0.66 lakh hectares; with a production of 4.92 lakh tonnes. The polyclonal, homestead system of banana cultivation prevalent in Kerala is unique, and the poor management in homesteads is one of the reasons attributed to the low productivity of banana in the State. 'Nendran' (Musa AAB 'Nendran') is the most popular dual purpose variety grown in Kerala. The incidence of bunchy top virus disease, and infestation of nematodes and other pests are the major production constraints of this variety.

Breeding of new banana varieties by sexual crossing is impeded by high sterility, polyploidy poor seed set and low germination percentage of hybrid seeds. In a crop like banana where natural variability is limited through continuous vegetative propagation, in vitro methods can be used with advantage to induce variability. Studies on mutation induction for genetic improvement of banana based on in vitro techniques were initiated at the International Atomic Energy Agency Laboratories at Vienna in Australia (Novak et al., 1989). Meristem tip culture is the widely used technique to regenerated in vitro plantlets for micropropagation and mutation breeding.

Callus proliferation and cell suspension; more recently, somatic embryos and subsequent regeneration of plantlets from seeded diploids have been achieved in banana (Novak et al., 1989). Formation of adventitious shoots by somatic organogenesis, especially through a callus phase increases the chances of variability (Skirvin and Janick, 1976). Similarly, variability can be achieved through repeated subculturing (Pierik, 1987).

Any genetic variation obtained in 'Nendran' variety through in vitro culture may be useful as selection could be made for desirable characters such as a shorter stature of the plant, higher yield, better colour of the bunches, lesser duration and more resistance to diseases and pests.

The present studies were undertaken to produce in vitro plantlets of Musa (AAB) 'Nendran' the most popular cultivar of Kerala, through the methods employed to induce variability namely, repeated subculturing, somatic organogenesis and embryogenesis.

Review of Literature

REVIEW OF LITERATURE

Clonal propagation of plant species through tissue culture is based on the concept of cell totipotency derived from the cell theory that Schleiden proposed in 1838. The origin of plant tissue culture could be traced to the earliest studies by Haberlandt (1902), who aseptically cultured single cells on a liquid medium supplemented with sugar.

In the recent years, remarkable progress has been made in the application of tissue culture methodology for mass clonal propagation of several economically important plant species. Genetic improvement of such crops will benefit the economy of the country (Moses, 1984). Cell culture techniques have been useful on several fronts in crop improvement programmes. An often cited advantage of cell culture is the provision for a more efficient means of selection to enhance the rate of recovery of desirable variants (Scowcroft, 1977).

Skirvin and Janick (1976) observed considerable variation among plants (calliclones) regenerated from cell cultures of scented geranium (Pelargonium sp.). They concluded that tissue culture techniques may have special uses for the plant breeder and should provide an additional method to increase intraclonal variation.

The term 'Somaclonal variation' has been coined to describe the variability commonly found among plants that have been regenerated from tissue culture (Larkin and Scowcroft, 1981). It appears to be a general phenomenon resulting from growth through a callus phase. Variability can also be induced by repeated subculturing (Pierik, 1987).

The micropropagation technique for cultivated Musa is well established (De Guzman et al., 1980; Cronauer and Krikorian, 1986; Jarret et al., 1985; Vuylsteke and De Langhe, 1985). Off-types produced by somaclonal variation have been reported in dessert banana Musa AAA group, 'Cavendish' (Reuveni et al., 1985; Hwang, 1986; Stover, 1987) and in plantain (Ramachandran et al., 1985). Such variation is significant in relation to the potential role of tissue culture in genetic manipulation and plant breeding programmes. In some cases, it may be possible to exploit this variation directly for breeding purposes; in other instances it may be an undesirable complicating factor. The applications of tissue culture techniques will, therefore, be influenced by the amount of variability or uniformity that can be expected in plants regenerated from cultured tissues or single cells. In banana, where the natural variability is limited by continuous clonal propagation, somaclonal variation can be used to advantage.

Maddock and Semple (1986) reported field assessment of somaclonal variation in wheat. More than 800 wheat lines derived from

plants regenerated in tissue culture have been grown in field trials comparable to those in the standard plant breeding programme. The majority of lines were healthy, uniform and similar to seed derived control material. However, examples of variation in the regenerated lines were also observed, the most noticeable differences being those in height and morphology.

In sugarcane, variants have been observed for disease resistance (eye spot disease, Fiji virus disease, downy mildew and smut), stalk number, sugar content, sugar yield and leaf traits, characters which are of concern in plant improvement. In Taiwan, Liu and Chen (1976) found that one particular somaclonal variant had a substantial improvement over its donor parent for both cane and sugar yield and, in addition, out-yielded Taiwan's best cultivar by 16 per cent.

Somaclonal variants in rice have been reported for characters such as number of fertile tillers per plant, average panicle length, frequency of fertile seed, plant stature and flag leaf length (Henke et al., 1978) among the regenerants from rice callus.

In banana, somaclonal variations have been induced by subjecting the explants to regenerate through a callus phase by somatic organogenesis/embryogenesis and through prolonged subculture. The relevant literature on this aspect is reviewed here.

2.1 Direct organogenesis through enhanced release of axillary buds

Swamy et al. (1983) observed that, shoot tips isolated from rhizomes of the banana cv. Robusta were suitable material for plantlet production in vitro. Excised shoot tips with the youngest leaves produced only one plantlet but shoot tips with several order sheathing leaf bases enclosing axillary buds, regenerated multiple plantlets.

Cronauer and Krikorian (1984) reported the establishment of rapidly multiplying cultures from excised shoot tips of two dessert banana clones and two plantain clones. Apices cultured on semisolid media produced single shoots while apices placed in liquid media produced shoot clusters. Individual shoots were induced to form multiple shoot clusters by splitting the shoot longitudinally through the apex. Shoot multiplication was stimulated by 5 mg BA per litre.

Aaquine (1989) observed a five fold multiplication rate every six weeks when lateral and terminal buds of cv. Giant cavendish was cultured on basal medium supplemented with 2 mg IAA and 3 mg BA per litre.

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; Swamy and Sahijram, 1989; Drew et al., 1989; Aravindakshan, 1989).

Cronauer and Krikorian (1985) observed reinitiation of vegetative growth from aseptically cultured terminal floral apex of banana. Terminal floral apices were isolated and cultured on modified MS medium (supplemented with - BA 5 mg per litre) and 10 per cent coconut water. Under these conditions, the determinate floral buds were transformed into multiplying vegetative shoot systems.

Swamy and Sahijram (1989) cultured excised floral apices of three banana cultivars viz. 'Chandrabale', 'Rasthali' and 'Robusta' on MS medium supplemented with cytokinins and auxins. The cultures reverted to vegetative state and produced a mass of green leafy shoots which were kept in a state of active growth by repeated subculturing. They had also found that the male flower clusters at different stages of development located on the peduncle subtending and distal to the meristematic zone reverted to vegetative state when cultured *in vitro*.

Drew *et al.* (1989) reported the applicability of floral apex as an explant for multiplying banana plants *in vitro*. For culture initiation MS medium supplemented with 160 g per litre adenine sulphate, 2 mg per litre IAA and 2 mg per litre kinetin was used. Shoot proliferation was achieved when MS medium was supplemented with 5 mg per litre BA.

2.1.1 Somaclonal variation through repeated subculturing

In banana much work has not been reported regarding this aspect.

A banana shoot stock (Colla AAA 'Dwarf Cavendish') was produced and maintained *in vitro* by means of successive subcultures (Rodriguez *et al.*, 1987). The propagation rate registered on subsequent subcultures was irregular and even in the same subculture each explant had different number of shoots. They concluded that this variability in the multiplication rate may be due to the difference in morphology and organogenic capacity of shoot structures, utilization of protocorm like shoot structures, and also due to shoots obtained from old stem fragments or from lateral buds.

2.2 Somatic organogenesis

Somatic organogenesis can be direct or callus mediated (Evans *et al.*, 1981). Although callus may be obtained from virtually any species, only in some can plants be regenerated. Even when totipotent callus has been obtained, extended proliferation by repeated subculturing may result in the reduction and eventual loss of the regenerative capacity. Reduction in shoot forming ability is often paralleled by an increase in the proportion of polyploid or aneuploid cells (Smith and Street, 1974).

The potential usefulness of callus in propagation lies in the

possibility of extending the base for adventitious shoots or embryo production. The principal disadvantage compared to clonal propagation is the genetic variation developing in many of the component cells. However regenerated variants can be used to complement the existing natural variability (Hussey, 1986).

Although several investigators have attempted to establish callus and subsequently, cell suspensions of bananas, this work has met with very limited success. Mohanram and Steward (1964), working the plugs of tissue from immature and mature but preclimateric fruits, obtained callus-like growth using a number of growth regulators. When these were transferred to liquid medium, slow growing suspensions were established. However, the culture proved to be non-morphogenic. Rao et al. (1982) have reported the establishment of callus cultures from discs of the inflorescence axis. The callus could be induced to form roots, but no shoot or bud formation was observed. When a single shoot is placed on the appropriate semi-solid medium, callus is formed at the shoot base. If liquid medium is used instead, the resulting cell suspensions 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, 1984). Bakry and Rossignol (1985) tried various explants of banana for analysing the callus formation and organogenesis; only floral or inflorescence tissues produced calli, but no organogenesis occurred.

Shoot tip was also taken as an explant in callus induction studies of banana (Jarret et al., 1985; Cronauer and Krikorian, 1986; Huang and Chi, 1988; Dhed et al., 1991).

Cronauer and Krikorian (1986) observed adventitious shoot production from calloid cultures of multiplying shoot cultures of triploid dessert clones (Philippine Lacatan, Grande Naine) cultured on MS media with added iron, sucrose, myoinositol and growth regulators. Round compact calli were produced by all cultures in the presence of 0.5 mg PCPA per litre. Although roots were induced by removing the kinetin, no shoots or plantlets were recovered.

Vigorous development of callus was achieved when the medium was supplemented with picloram and solidified with gelrite (Huang and Chi, 1988) which also eliminated tissue and medium discolouration. A small proportion of the callus cells displayed the normal chromosome number 2n = 33, the majority being variable aneuploids.

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

2.3 Somatic embryogenesis

Sharp et al. (1982) reported the application of somatic

embryogenesis for crop improvement. Progress in the release of improved crop varieties has been facilitated by developing protocols for high frequency somatic embryogenesis. Two general patterns in vitro embryo development were reported, (1) Direct embryogenesis from tissues, marked by absence of callus proliferation and (2) Indirect embryogenesis in which callus proliferation occurs prior to embryo development. Direct embryogenesis proceeds from pre-embryogenic determined cells while indirect embryogenesis requires the redetermination of differentiated cells, callus proliferation and differentiation of induced embryogenic determined cells (I.E.D.C.). Somatic embryogenesis will be useful for the improvement of crops and for the recovery of novel genotypes.

Somatic embryogenesis was clearly described in carrot (Reinert, 1959; Halperin and Whetherell, 1964). Reviews 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-Purnel, 1990; Dhed et al., 1991).

Cronauer and Krikorian (1983) observed somatic embryos from cultured tissues of triploid plantains (Musa ABB). Somatic embryoids of triploid clones, Saba and Pelipita were produced in liquid culture medium from cells derived from multiplying shoot tip cultures. Green somatic embryoids formed only in medium containing 2,4,5-T.

Banerjee *et al.* (1985) observed somatic embryogenesis in *Musa*. Thin meristematic layers excised from proliferating shoot tip cultures of *Musa* ABB cv. Bluggoe produced callus when cultured on MS basal medium supplemented with other auxins and cytokinins. The proembryoids obtained from 2,4-D containing medium produced only roots, while those from 2,4,5-T containing medium regenerated bipolar embryoids.

Fitchet (1987) observed somatic embryogenesis in callus of Dwarf Cavendish banana. The rate of proliferation and friability of the callus produced increased with the number of subcultures. The callus was maintained on the same medium and transferred at monthly intervals, as a result numerous embryoids and single plantlets have been produced.

Novak *et al.* (1989) obtained somatic embryogenesis and plant regeneration in suspension cultures of dessert and cooking bananas. Proembryogenic calli were initiated from basal leaf sheaths and rhizome tissue on modified Shenk and Hildebrandt (SH) medium with 30 μM 3,6-dichloro-2-methoxy benzoic acid (Dicamba).

Fitchet-Purnel (1990) obtained embryo like structures from banana suspension cultures. The explants were taken from plantlets produced *in vitro* from pseudostem apices. Attempts to induce further development of the embryos into plants were unsuccessful.

Plant regeneration was obtained (Dhed· et al., 1991) when embryogenic cell suspension cultures were established from meristems cut from proliferating cultures of shoot tips. Embryogenic globules matured and germinated in the presence of BA or zeatin. Plantlets were then transferred to semisolid medium and eventually to soil.

2.4 In vitro cytological changes

There are several mechanisms which might give rise to somaclonal variation (Skirvin, 1978; Chaleff, 1981; Shepard, 1981; Larkin and Scowcroft, 1981). These include ploidy changes, nuclear fragmentation, inter and intra chromosomal inter changes, somatic crossing over and sister chromatid exchanges, gene amplification and transposable genetic elements.

Evidences from cytological and molecular studies show that the plant genome is relatively unstable and subject to various changes in developing somatic tissues. Larkin and Scowcroft (1981) reported numerical or structural changes in chromosomes in association with in vitro regeneration of plants. Numerical changes have been observed in callus cultures of several crops (Evans and Sharp. .1983).

According to D'Amato (1975), polyploidy· occured in the differentiated tissues of 90 per cent of all plant species. Gene amplification has been shown to occur in plants grown in stressful

environments (Cullis, 1975). More than 75 per cent of DNA sequences consists of repetitive DNA which is particularly susceptible to change (Flavell, 1984). The variability reflected either pre-existing cellular genetic differences or tissue culture induced variability (Evans et al., 1984).

D' Amato (1984) studied nuclear cytology of tissue cultures. When meristems or other plant explants were cultured on hormone containing media, callus may be formed and genetic variation would arise. Callus and suspension cultures were reported to be reservoirs of variation, whose extent partially depended on external factors (especially the hormone component of the medium, type of culture and culture regime), and partially on intrinsic factors (eg. genetic constitution of the species, origin of the explant).

Variation in chromosome structure and number in plant cells during in vitro tissue culture was investigated by Singh (1984). In banana, minute chromosome size as well as low regeneration potential of the callus has limited the cytological evaluation.

In Pinus sp. chromosomal variation in the callus was studied by Kim and Park (1986). Excised mature embryos of Pinus koraiensis were plated on a media supplemented with growth regulators - 0.1 mg per litre NAA (M-1); 0.1 mg per litre NAA and 2,4-D (M-2); 0.1 mg per litre 2,4-D and BAP (M-3), 0.1 mg per litre 2,4-D and kinetin (M-4). Cytological study of the callus showed that the

occurrence of diploid cells was observed to a level of 52 per cent in M-3 and 36 per cent in M-4 media. The stable chromosome state is a crucial factor for organogenesis. Therefore, it can be inferred that the callus tissues cultured on media supplemented with both auxin and cytokinin have the greatest possibility of organogenesis.

2.5 Field evaluation of somaclonal variation

Field evaluation of *in vitro* grown banana was done by several workers (Ventura *et al.*, 1988; Vuylsteke *et al.*, 1988; Robinson, 1989; Robinson and Nel, 1989; Robinson and Anderson, 1990; Vuylsteke and Swennen, 1990 and Israeli *et al.*, 1989).

Ventura *et al.* (1988) observed somaclonal variation in micro-propagated bananas. Clones of genomic group ABB were evaluated in the field. Among the plants, 10.4 per cent showed somaclonal variation in bunch characters (number of hands per bunch, shorter fruits, increased fruits per bunch and atrophied fruits) and 19.6 per cent showed variation in plant height.

Plants derived from a clone Americani (group AAA, sub group Cavendish) included variants which ranged in height between Dwarf Cavendish and Grande Naine.

Vuylsteke *et al.* (1988) found phenotypic variation among *in vitro* propagated plantain (*Musa* sp. cultivar AAB). Observations of the first crop and the successive ratoon indicated five different

cycle time. The tissue cultured plants were taller with a greater stem circumference and vigour.

Vuylsteke and Swennen (1990) observed variation in inflorescence types in African plantains. Along with variation in inflorescence, they exhibit variation in degree of female fertility, pseudostem characters, petiole and bract colour, leaf and growth habit. Much of this somaclonal variation was enhanced *in vitro*.

Quantitative aspects of somaclonal variation was reported by Israeli *et al.* (1991). Somaclonal variants of seven *in vitro* propagated banana cultivars were expressed in plant stature, abnormal leaves, pseudostem pigmentation, persistance of flowers and split fingers. Dwarfism was the most common variant indicated by mutation of intermediate height cultivars to a dwarf stature and in one case of a iow statured cultivar to an extra dwarf stature. The second most common group of variants were characterised by different degrees of thick and rubbery narrow leaves with variable pale green mottling (mosaic). These variants observed in the Cavendish sub group and in cv. Red were suspected to be aneuploids.

Materials and Methods

MATERIALS AND METHODS

The present investigations on the induction of genetic variability in Musa sp. var. Nendran by in vitro methods, were carried out in the Department of Pomology and Floriculture and the Plant Tissue Culture Laboratory attached to the All India Co-ordinated Floriculture Improvement Project (AICFIP), College of Horticulture, Vellanikkara during 1991-93. The details regarding the experimental methods and analytical techniques adopted are presented in this chapter.

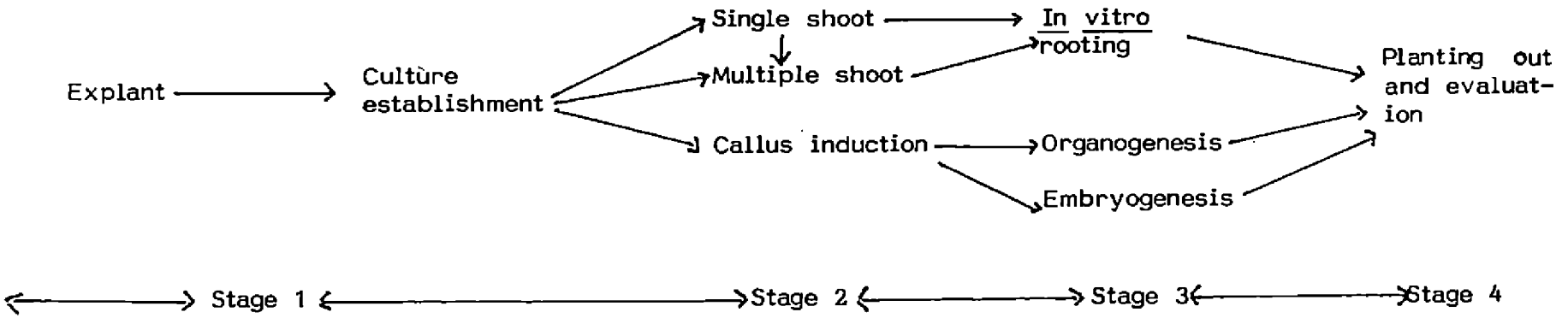
In the present study the explants from the banana variety Nendran (Musa AAB 'Nendran') were regenerated through a callus phase by somatic organogenesis/somatic embryogenesis and through prolonged subculture cycles. The different stages involved are illustrated in Fig.1.

3.1 Selection of explant

The explants used for the study and the routes tried are given below.

Route	Explant
Enhanced release of axillary buds	Shoot tip (the main apical bud collected from a five month old sword sucker of banana).
	Eye bud (the main apical bud collected from the small buds seen on the sides of the mother rhizome of banana).

Fig. 1. In vitro cloning procedure in banana



Stage 1. Physiological preconditioning of the explant and explant establishment

Stage 2. Induction of axillary shoots/callus mediated organogenesis and rapid multiplication

Stage 3. In vitro rooting and acclimatization

Stage 4. Planting out and evaluation

Somatic organogenesis/ embryogenesis	Shoot tip (the main apical bud collected from a five months old sword sucker of banana) Flower base Inflorescence axis Embryonic leaves Scalp (Meristem isolated from proliferating shoot tip)

3.1.1 Collection and preparation of explant

Shoot tip explants for enhanced release of axillary buds were collected from five month old sword suckers grown in the field. Suckers after separation from the mother rhizome were detopped and were reduced to a size which measured about 5 cm in length retaining a small portion (1 cm length) of the rhizome tissue. In the case of eye bud explants, small buds seen on the surface of the mother rhizome were removed using a sharp knife without injuring the central bud, also retaining a small portion of the rhizome tissue.

Shoot tip, flower base, inflorescence axis, embryonic leaves and the scalp were the explants used for induction of somatic organogenesis. Collection and preparation of shoot tip explants was same as in the case of enhanced release of axillary buds.

For inflorescence axis explants, the male bud were collected from the distal end of the peduncle after the completion of the

female phase of the bunch. The subtending bracts of the floral apex with their associated hands of female flowers were removed one by one in a stepwise manner until they became too small to be removed by hand. Tender parts of the inflorescence axis were used for culturing. In the case of flower base explants also the male buds were collected, and the subtending bracts of the floral apex were removed one by one, until the male bud became small. The tender flowers were then selected. Embryonic leaves were collected from 3-5 month old peepers. Scalp explants were collected from proliferating shoot tips under culture (in vitro).

The explants (except scalp) after collection were immediately taken to the laboratory, where they were washed thoroughly in tap water to remove all the dirt and soil particles adhering to them. After drying them in between folds of tissue paper, they were again washed with sterile distilled water, further sterilization procedures were carried out under perfect aseptic conditions in a 'Klenzaids' laminar air flow cabinet. The explants except scalp were then subjected to surface sterilization using different chemicals as given in Table 1. As the scalp tissues were taken from in vitro grown shoot tips, they were not subjected to surface sterilization.

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

Table 1. Treatments given for surface sterilization of explants

Explant	Sterilant	Concentration (%)	Duration (minutes)
Shoot tip and eye bud	Mercuric chloride	0.1	10
	,,	0.1	20
	,,	02	10
	Emisan	0.1	20
	,,	0.1	30
	Emisan +	0.1	30
	Norfloxacin +	0.2	10
	Mercuric chloride	0.1	20
		0.1	30
	,,	0.1	20
		0.1	20
		0.1	30
	,,	0.1	30
		0.1	20
Flower base, Embryonic leaves and Inflorescence axis	Emisan	0.1	10
	,,	0.1	20
	,,	0.1	30
	Alcohol	70.0	1 sec
	,,	70.0	1sec

Sterilized shoot tips were further reduced in size by trimming down the cut surface of the rhizomatous tissues and leaf bases, using sterilized scalpel and forceps. Inflorescence explants were sliced into pieces of about 0.5 cm in length and 1 cm in breadth before inoculation. In the case of flower base explants, tender flowers were selected from male buds after sterilization and the base of the flower was excised from the other parts using a scalpel. For culturing of leaves, sections of about 5 mm were isolated and inoculated into the culture medium. Scalp tissues were excised from proliferating shoot tips using a needle and forceps (sterilized).

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

3.2 The media

The culture media used for the study was MS (Murashige and Skoog, 1962), SH (Shenk and Hildebrandt, 1972) and LS (Linsmaier and Skoog, 1965). The chemical composition of the media is given in Table 2.

The chemicals used for preparing the culture media were of analytical grade from British Drug House (BDH), Sisco Research Laboratories (SRL), Merck or Sigma.

(AAB) 'Nendran' tissue culture

Ingredients	Amount (mg/l)		
	MS[a*]	SH*	LS*
Macronutrients			
KNO_3	1900.0	2500.0	1900.0
NH_4NO_3	1650.0	-	1650.0
$(NH_4)_2SO_4$	-	-	-
KH_2PO_4	170.0	-	170.0
$NaH_2PO_4.H_2O$	-	-	-
$NH_4H_2PO_4$	-	300.0	-
$MgSO_4.7H_2O$	370.0	400.0	370.0
$CaCl_2.2H_2O$	440.0	200.0	440.0
Micronutrients			
H_3BO_3	6.200	5.00	6.200
$MnSO_4.H_2O$	-	10.00	-
$MnSO_4.4H_2O$	22.300	-	22.300
$ZnSO_4.7H_2O$	8.600	1.00	8.600
$NaMoO_4.2H_2O$	0.250	0.10	0.250
$CuSO_4.5H_2O$	0.025	0.20	0.025
$CoCl_2.6H_2O$	0.025	0.10	0.025
KI	0.830	1.00	0.830
$FeSO_4.7H_2O$	27.800	15.00	27.800
Na_2EDTA	33.600	20.00	33.600
Vitamins			
Thiamine.HCl	0.10	5.00	-
Pyridoxine.HCl	0.50	0.50	-
Nicotinic acid	0.05	5.00	-
Others			
Glycine	2.00	-	-
Myo-inositol	100.00	1000.00	-
Sucrose	30.00	30.00	-
pH	5.80	5.50	-

*MS - Murashige and Skoog (1962)
*SH - Shenk and Hildebrandt (1972)
*LS - Linsmaier and Skoog (1965)

Schenk

*MS[a] - Murashige and Skoog media containing full concentration of inorganic salts and organic growth factors

Standard procedure (Gamborg and Shyluk, 1981) were followed for the preparation of MS media. Stock solutions of major and minor nutrients were prepared first by dissolving the required quantity of chemicals in double distilled water and were stored under refrigerated conditions in amber coloured bottles. The stock solution of nutrients were prepared fresh every four weeks and that of vitamins, amino acids and growth regulators were prepared fresh every week.

Specific quantities of the stock solutions of chemicals and growth regulators were pipetted out into a 1000 ml beaker. Sucrose and inositol were added fresh and dissolved. The volume was then made upto about 1000 ml by adding double glass distilled water. The pH of the solution was adjusted using an electronic pH meter. The pH was adjusted in between 5.5-5.8. Agar was then added to the medium and the final volume was made up exactly to 1000 ml.

For the preparation of LS media, specific quantities of macro and micro nutrients were pipetted out from the stock and required quantities of growth regulators and sucrose were added into the same, volume was made upto about 1000 ml by adding double distilled water. The pH of the solution was adjusted between 5.5-5.8. Agar was then added to the medium and the final volume was made up exactly to 1000 ml.

SH media was prepared by weighing out required quantities of major and minor nutrients and were dissolved in double distilled water, growth regulators and sucrose were added, then the volume was made upto about 1000 ml by adding double distilled water. The pH of the solution was adjusted between 5.5-5.8. Agar was then added to the medium and the final volume was made up exactly to 1000 ml.

The solutions were then melted by keeping in a water bath, maintained at a temperature of 90-95°C. The medium (@ 15 ml) was poured hot to the oven sterilized culture vessels which were previously rinsed twice with double distilled water. The containers with the medium were then tightly plugged with nonabsorbent cotton wool plugs. Borosil brand test tubes and corning brand conical flasks were the containers used.

In order to ensure aseptic condition of the medium, the containers plugged with cotton were autoclaved for 15-20 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 Preparation and inoculation of explants

All the inoculation operations were carried out under perfect aseptic conditions in a 'Klenzaids' laminar air flow cabinet.

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

The culture vessels were then transferred to a culture room where they were incubated at a temperature of 27±2°C. Artificial illumination was provided using cool white fluorescent lamps. The light intensity was maintained at 2000 lux. Photoperiod was fixed as 16 h per day which was regulated by a diurnal timer.

To reduce media and explant discolouration, different treatments were given to explants, the details of which are given below.

Treatment	Levels
1. Adding ascorbic acid into the media	15, 30, 50 mg per litre
2. Adding activated charcoal into the media	250, 500, 750 mg per litre

Observation on the percentage of cultures without media and explant discolouration were made on ten explants per treatment after four weeks of culturing.

3.4 Enhanced release of axillary buds

3.4.1 Culture establishment (Stage 1)

In order to study the morphogenic response of banana explants in culture, the most widely accepted MS medium (Murashige and Skoog, 1962) was tried (Table 6 and 7). This medium contained only the basal salts (macro and micro), vitamins and sucrose as reported in the original publication. This was supplemented with cytokinins and auxin at various concentrations.

Explant	Treatments
Shoot tip	4 x 2 combinations of BA (2.5, 5, 7.5, 10 ppm) and NAA (1, 2 ppm)
Eye bud	4 x 2 combinations of kinetin (2.5, 5, 7.5, 10 ppm) and NAA (1, 2 ppm)

Observations on the number of days taken for culture establishment and culture elongation were made after four weeks of culturing.

3.4.2 Culture proliferation (Stage 2)

All the trials on stage 2 were carried out on MS (1962) media. Explants used for induction of axillary shoots were 30 mm long shoots from establishment culture. The details of the trials on standardisation of basic proliferation medium utilising growth regulating substances viz. cytokinins and auxin are presented below.

Explant	Treatment
Shoot tip	MS^a + 3 x 2 combination of BA (5, 7.5, 10 ppm) and NAA (1, 2 ppm)
Eye bud	MS^b + 3 x 2 combination of BA (5, 7.5, 10 ppm) and NAA (1, 2 ppm)

Observations on percentage of cultures developing shoots and number of shoots produced per culture were recorded on three replications after four weeks of culturing.

3.4.2.1 Effect of continuous subculturing on the multiplication rate

Regular subculturing of the proliferated shoots on to medium containing high concentrations of BA was found to increase the number of shoots per culture. A trial was conducted to assess the multiplication rate of shoots, on continuous subculturing at two weeks interval in ten serial subcultures. Semi-solid MS medium containing BA 5 ppm and NAA 1 ppm and cultures derived from shoot tip explants of 'Nendran' were used for the study. Observations were recorded on three replications per treatment on the number of shoots produced per culture and the percentage increase in number of shoots over the initial culture.

3.4.2.2 Proliferation of calloid

Calloid (hard, greenish globular structure which resemble callus) obtained from shoot proliferation media (MS^b + BA 10 ppm)

was subcultured into different combinations of auxin and cytokinins for further proliferation.

Explant	Treatment
Calloid	MS^a + BA 5 ppm + NAA 1 ppm
	,, + KIN 5 ppm + NAA 1 ppm
	,, + basal
	MS^b + BA 5 ppm + NAA 1 ppm
	,, + KIN 5 ppm + NAA 1 ppm
	,, + basal

Observations were taken after four weeks of culturing on the number of buds/culture and percentage of culture producing shoots.

3.4.3 In vitro rooting and acclimatization (Stage 3)

3.4.3.1 In vitro rooting

All the trials on in vitro rooting were conducted on half and full strength MS medium. Shoots (2 to 3 cm length) excised from shoot proliferating cultures were used for these trials. AC @ 0.05 per cent was a common factor in all treatments. The different levels of auxin (NAA) tried for rooting of banana shoots were 2, 5 and 10 ppm.

Observations on the percentage of cultures showing root initiation, number of days required for root initiation and the number of roots produced per shoot were recorded.

3.5 Somatic organogenesis

3.5.1 Culture establishment (Stage 1)

3.5.1.1 Effect of media

For callus initiation, experiments were carried out using different media and explants under various conditions as given below.

Culture condition	Media	Explant	Treatment
Light	MS[a] semi solid	Embryonic leaves Scalp (*in vitro*)	2,4-D 5 ppm + BA 1 ppm
	Liquid		
	MS[b] semi solid.		
	Liquid		
	SH semi solid		
	Liquid		
	LS semi solid		
	Liquid		
Dark	MS[a] semi solid		
	Liquid		
	MS[b] semi solid		
	Liquid		
	SH semi solid		
	Liquid		
	LS semi solid		
	Liquid		

Observations on percentage of culture survival, time taken for callus initiation and percentage of culture initiating callus were recorded after four weeks of culture.

3.5.1.2 Effect of explants

Explants (scalp and embryonic leaves) were cultured in MS^b medium supplemented with various concentrations of auxin and cytokinin which are presented below.

Media	Treatments
MS^b	2,4-D (5, 7 ppm) + BA (1 ppm)
	2,4,5-T (5 ppm) + BA (1 ppm)

Observations were recorded on the response of explant on culture establishment after four weeks of culturing, as the mean of ten cultures.

3.5.2 Culture proliferation (Stage 2)

The treatments tried for the study is given below.

Media	Treatments
MS^a and MS^b	4 x 1 level of 2,4-D (5, 7, 2, 1 ppm) and BA (1 ppm)
	3 x 1 level of 2,4,5-T (5, 2, 2.5 ppm) and BA (1 ppm)

All the cultures were kept under illuminated conditions. The explants showing response in the culture establishment were used for the study. The following observations were recorded.

3.5.2.1 Percentage of cultures initiating callus (P)

After four weeks of culturing, the number of cultures showing response out of five cultures was noted and recorded as percentage.

3.5.2.2 Growth of the callus (G)

It was assessed based on a visual rating (with score 1 to the smallest and score 4 to the largest callus). The mean score was expressed as the growth score.

3.5.2.3 Callus index (CI)

It was computed by multiplying per cent explants initiating callus (P) with the growth score (G).

3.5.3 Callus differentiation

Explants which produced callus were subcultured into the callus differentiation media. Half and full MS media were used as media, supplemented with 3 levels of 2,4-D (1, 2, 5 ppm) 3 levels of BA (1, 5, 10 ppm) and the basal media. Both liquid and semi-solid consistency of the medium was tried. Response was noted after four weeks, values were taken as the mean of five cultures.

For callus differentiation, MS media was supplemented with varying levels of nitrate source (NH_4NO_3 - 1.65 g, KNO_3 - 1.90 g was changed to $\frac{1}{2}$, full and $1\frac{1}{2}$ the quantity present in the media). Response was noted as the mean of five cultures after four weeks.

3.6 Somatic embryogenesis

As maximum callusing was noted from scalp, this was taken as the explant for somatic embryogenesis. The method described by Dhed et al. (1991) was followed (Fig. 2).

3.6.1 Culture establishment (Stage 1)

Explants were cultured in MS^b (liquid) medium supplemented with 2,4-D 5 ppm and BA 1 ppm.

Observations were recorded on the response of explant on culture establishment after four weeks of culturing, as the mean of ten cultures.

3.6.2 Callus initiation and embryogenesis

Cell suspensions have been initiated from cv. Nendran using explants excised from the upper part of the proliferating meristematic shoot tips. The explants (scalps) were inoculated in liquid medium with the mineral salts of Murashige and Skoog, but with half strength macroelements and iron, 3 per cent sugar, 5 uM BA. The cultures were maintained at 26±2°C under 16 h light. Then suspension in maintanence was sieved and after sedimentation, 1 ml transferred into liquid medium devoid of growth regulators and containing 100 mg inositol. Embryonic globules were transferred to a medium containing cytokinin (MS + BA 1 ppm).

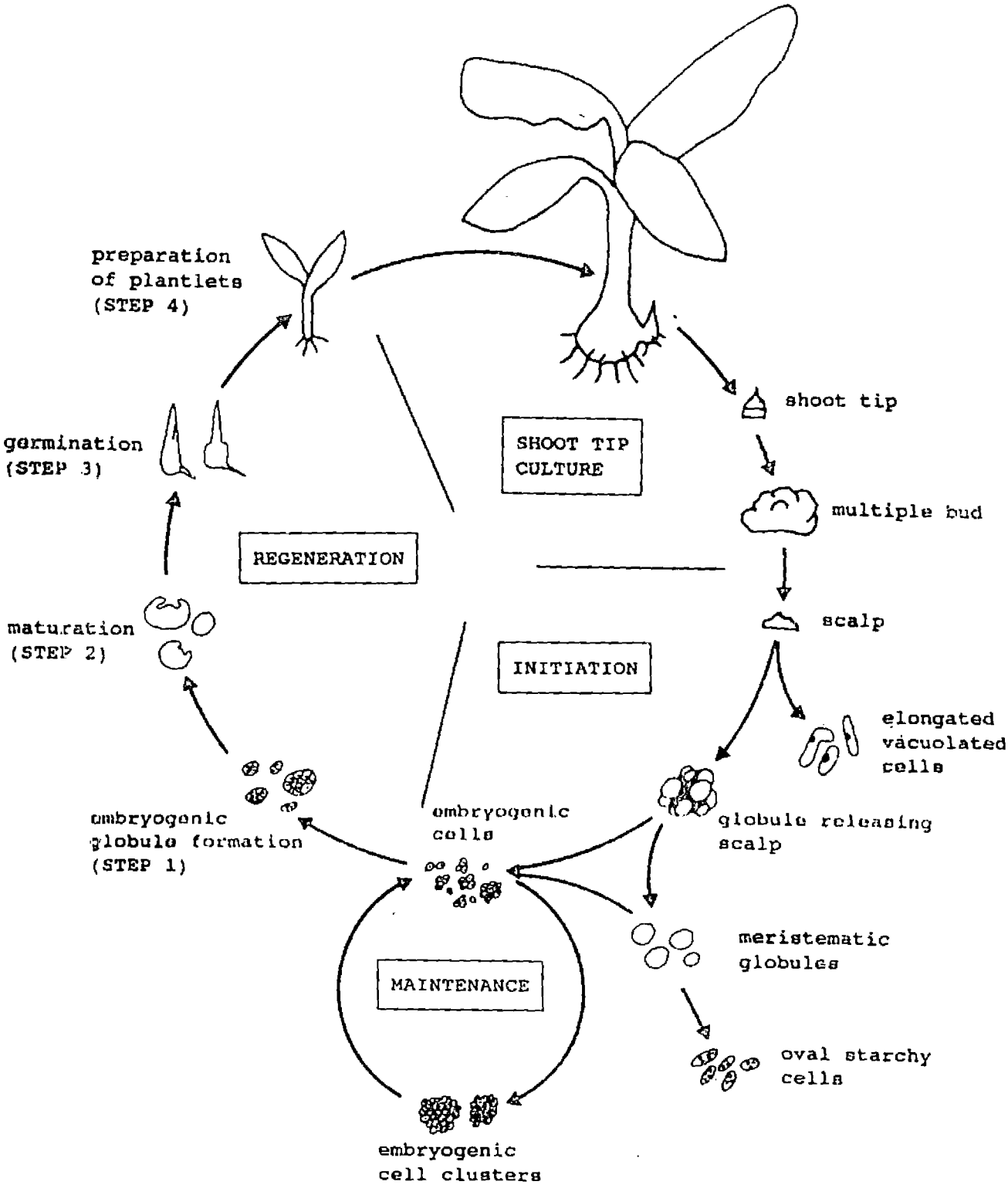


Fig. 2. Digramatic representation of Musa cell suspension culture and the regeneration of plants therefrom (Dhed et al., 1991)

3.7 Planting out and hardening

Hardening treatments for *in vitro* banana plants were already standardised in the previous study conducted by Bhaskar (1991). The best treatment among them was adopted for the hardening of the developed plantlets. In this method, the control of temperature and maintenance of relative humidity during acclimatization was also achieved by covering the plantlets with transparent plastic microscope covers having 60 cm height and 45 cm diameter at the base. Spraying of cold water (12±2°C) at an interval of three hours during day time using a hand sprayer with fine mist nozzle enabled to maintain high relative humidity (90-100 per cent) and mild temperature (26±3°C) inside the plastic cover.

As the pretransfer hardening treatment, the plantlets were kept in distilled water for 8 h followed by 8 h in MS liquid medium under microscope cover.

The following observations were recorded on the growth parameters of *in vitro* grown banana plantlets at the time of planting.

3.7.1 Plant height

Length from collar region to the tip of the plantlets was recorded and expressed in cm.

3.7.2 Number of leaves per plant

The total number of leaves produced by a plantlet was counted and recorded.

3.7.3 Length of the longest leaf

Length was taken from the base to the tip of the leaf and expressed in cm.

3.7.4 Number of roots per plant

The total number of roots per plantlet was counted and recorded.

3.7.5 Average length of the root

Length of the root was measured from the collar region to the tip and the mean length was expressed in cm.

3.8 Containers and media

3.8.1 Containers

Black plastic cups were used as containers for planting out the plantlets.

3.8.2 Potting media

In order to study the effect of the potting media on the growth of plantlets the plantlets were planted in different potting media.

1. Sand
2. Perlite : Manufactured by KEL Perlite, Bangalore
3. Soilrite : A mixture of peat moss, vermiculite and perlite marketed by KEL Perlite, Bangalore
4. Cocopeat : A proprietary compound containing decomposed peat and coir dust produced by Langalee Ltd., Bangalore

Biofibe : Peat free soil less growing mix manufactured by Red Sander's, Bangalore

The potting media after wetting were autoclaved at 15 psi for 30 min to make it free from soil born pathogens. Small plastic bowls with sufficient holes for drainage were used for filling the media. The containers were drenched with a weak solution (0.05 per cent) of Bavistin. The plantlets were then planted in the potting media and subjected to post planting treatments.

Observations of plantlets grown in different potting media were recorded on characters such as the percentage of survival, height of the plant, total number of leaves, days taken for leaf production after transplanting and the length of the longest leaf. Observations were taken at 10 days interval, and a total of four observations were taken, as the mean of four plantlets in each potting media.

3.9 Assessment of somaclonal variation

In order to study the morphological variations among the plantlets derived from different subcultures, they were maintained separately in different plastic pots. Sterile sand was used as the medium. Observations like survival percentage, height of the plant, number of leaves, length of the leaves, breadth of the leaves and the girth of the plantlets at weekly intervals were recorded.

3.9.1 Cytological evaluation

Root tips from *in vitro* plantlets of different subcultures were excised for the cytological evaluation of banana plants which was carried out to identify whether any change in chromosome number has arisen due to repeated subculturing.

Root tips were excised from the plantlets just before transplanting between 7.30 am - 8.00 am. Root tips were then kept in a fixative (alcohol : acetic acid in 3 : 1 ratio). After 8-10 h, they were taken out, washed in distilled water and kept for hydrolysis in 1 N HCl for 10 min at 60°C. After hydrolysis they were again washed and finally kept in a staining solution (fulgen stain) for an hour. Squash preparations of the root tips were then observed under a microscope, and the somatic chromosomes were counted.

3.9.2 Growth parameters

Plantlets from different subcultures were planted out in sand, which was found to be the best medium and observations were recorded at 15 days interval on growth parameters such as the plant height, girth, number of leaves, length and width of the 3rd leaf and the variations in these morphological characters were noted. From the observations, the difference in growth rate with respect to plant height and leaf area were also found.

3.10 Statistical analysis

The data from the various experiments were statistically analysed as per Panse and Sukhatme (1985).

Results

RESULTS

The results of the investigations on the induction of variability in Musa (AAB) 'Nendran' by in vitro methods, are presented in this chapter.

4.1 Surface sterilization of explants

The data on the surface sterilization of two types of explants viz., shoot tip and eye bud using various sterilants are presented in Table 3 and the data on the surface sterilization of the other three types of explants viz., flower base embryonic leaves and inflorescence axis are presented in Table 4.

In the case of shoot tip explants, of the various sterilants tried, treatment with norfloxacin (0.1 per cent) for 30 minutes, gave better sterilization than mercuric chloride and/or emisan alone. An initial dipping of shoot tip explant in emisan (0.1 per cent) for 30 minutes, followed by dipping in norfloxacin (0.1 per cent) for 30 minutes and finally rinsing of shoot tip in mercuric chloride (0.1 per cent) for 20 minutes resulted in the least rate of contamination (11.66 per cent) and the maximum percentage of explant survival (88.33 per cent).

For eye bud explants, an initial dipping in emisan (0.1 per cent) for 30 minutes followed by dipping in norfloxacin (0.1 per cent) for 30 minutes and finally in mercuric chloride (0.1 per cent)

Table 3. Standardisation of surface sterilization methods for shoot tip and eye bud explants of Musa (AAB) 'Nendran'

Basal medium - MS[a]

Sterilant	Concentration (%)	Duration (minutes)	Shoot tip		Eye bud	
			Contamination (%)	Survival (%)	Contamination (%)	Survival (%)
1. Mercuric chloride	0.1	10	100.00		100.00	-
2. Mercuric chloride	0.1	20	53.33	46.66	58.33	41.67
3. Mercuric chloride	0.2	10	100.00		100.00	-
4. Emisan	0.1	20	65.00	35.00	71.66	28.34
5. Emisan	0.1	30	55.00	45.00	61.66	38.34
6. Emisan + Norfloxacin + Mercuric chloride	0.1 0.1 0.1	30 10 20	45.00	55.00	33.33	66.67
7. Emisan + Norfloxacin + Mercuric chloride	0.1 0.2 0.1	30 10 20	38.33	61.66	51.66	48.34
8. Emisan + Norfloxacin + Mercuric chloride	0.1 0.1 0.1	30 20 20	26.66	73.33	43.33	56.67
9. Emisan + Norfloxacin + Mercuric chloride	0.1 0.1 0.1	30 30 20	11.66	88.33	20.00	80.00

Table 4. Standardisation of surface sterilization methods for flower base, embryonic leaves and inflorescence axis of Musa (AAB) 'Nendran'

Basal medium - MS[a]

Sterilant	Concentration (%)	Duration	Flower base		Embryonic leaves		Inflorescence axis	
			Contamination (%)	Survival (%)	Contamination (%)	Survival (%)	Contamination (%)	Survival (%)
Emisan	0.1	10 min	100	0	81.7	18.3	100.00	0.00
Emisan	0.1	20 min	0	100	70.0	30.0	51.67	48.33
Emisan	0.1	30 min	90	10	100.00	0.0	100.00	0.00
Alcohol	70.0	1 sec	100	0	20.0	80.0	100.00	0.00
Alcohol	70.0	2 sec	100	0	100.0	0.0	100.00	0.00

Culture period - Four weeks

Values taken as average of four observations

[a]MS medium containing full concentration of both inorganic salts and organic growth factors

for 20 minutes resulted in the least rate of contamination (20 per cent) and the maximum percentage of explant survival (80 per cent).

The results of the surface sterilization of flower base, embryonic leaves and inflorescence axis explants indicated that (Table 4) among the different concentrations of emisan and alcohol tried for flower base explants, treatments with emisan (0.1 per cent) for 20 minutes recorded maximum survival (100 per cent). For inflorescence axis explants also, the same treatment resulted in maximum survival (48.33 per cent) and minimum contamination (51.67 per cent). In the case of embryonic leaves, dipping in alcohol for one second recorded maximum survival (80 per cent) and minimum contamination (20 per cent).

4.2 Discolouration of media and explants in in vitro culture of banana

Addition of activated charcoal (500 mg per litre) to the medium, resulted in maximum percentage of culture survival (42.00) and maximum percentage of culture, without media and explant discolouration (100), than all other treatments (Table 5). Also it was observed that the treatment was on par with ascorbid acid 50 mg per litre. Among the different concentrations of ascorbic acid tried, 50 mg per litre was found to be better for maximum culture survival (31 per cent) and minimum media and explant discolouration (22.33 per cent) than the other treatments.

Table 5. Effect of medium supplements on the discolouration of media and explant in in vitro culture of Musa (AAB) 'Nendran'

Media - MS[a*] + BA 5 ppm + NAA 2 ppm

Explant	Treatments	Concentration (mg/l)	Death of culture due to contamination (%)	Death of culture due to discolouration (%)	Survival (%)	Nature of discolouration
Shoot tip	Ascorbic acid	15	61.67	38.33	0.00	Explant and media turned dark brown colour
Shoot tip	Ascorbic acid	30	47.33	34.70	18.00	,,
Shoot tip	Ascorbic acid	50	43.33	22.33	31.00	,,
Shoot tip	Activated charcoal	250	50.00	46.67	3.33	Partial darkening of explant and media
Shoot tip	Activated charcoal	500	58.00	0.00	42.00	No discolouration
Shoot tip	Activated charcoal	750	68.33	3.33	28.33	Slight darkening of the explant
CD (0.05)			5.9	10.13	9.93	
SEm±			2.7	4.67	4.58	

Culture period - Four weeks
Average of three replications
*MS medium containing full concentration of both inorganic salts and organic growth factors

4.3 Enhanced release of axiliary buds

4.3.1 Culture establishment (Stage 1)

In order to standardise a suitable culture establishment medium, trials were conducted with different levels of cytokinins (BA and kinetin) and auxin (NAA) in MS (semi-solid) medium using excised shoot tip and eye bud explants. All the explants which were not contaminated turned green within a period ranging from 8 to 29 days depending upon the kind and concentration of growth regulators and the type of explant used. The results of the experiments are presented below.

4.3.1.1 Effect of BA and NAA

Establishment of the cultures occurred in all the eight combinations of NAA and BA (Table 6). Shoot tip explants took 8.33 to 24.33 days and eye bud explants 14.33 to 26 days for culture establishment. The treatment combination involving NAA 2 ppm + BA 5 ppm was significantly superior to all other treatments. Under this treatment, shoot tip and eye bud explants took minimum number of days (8.33 and 14.33, respectively) for culture establishment. The treatment was on par with that of media with NAA 2 ppm + BA 7.5 ppm, in which culture establishment occurred within 10.33 and 16.33 days for shoot tip and eye bud explants respectively.

Culture elongation occurred in all the eight treatments. Shoot tip explant took 16.33 to 32.33 days and eye bud explant 21 to

Table 6. Effect of NAA + BA on culture establishment of shoot tip and eye bud explants of Musa (AAB) 'Nendran'

Basal medium - MS[a*]

Treatments	Shoot tip		Eye bud	
	Time taken for culture establishment (days)	Time taken for culture elongation (days)	Time taken for culture establishment (days)	Time taken for culture elongation (days)
NAA 1 ppm + BA 2.5 ppm	24.33	32.33	26.00	33.00
NAA 1 ppm + BA 5 ppm	22.33	28.00	21.66	28.67
NAA 1 ppm + BA 7.5 ppm	16.33	22.33	23.00	30.00
NAA 1 ppm + BA 10 ppm	13.00	23.00	21.66	29.33
NAA 2 ppm + BA 2.5 ppm	18.33	27.00	20.66	26.33
NAA 2 ppm + BA 5 ppm	8.33	18.33	14.33	21.00
NAA 2 ppm + BA 7.5 ppm	10.33	16.33	16.33	22.33
NA 2 ppm + BA 10 ppm	13.33	18.66	19.66	26.33
CD (0.05)	2.81	1.79	2.73	2.33
SEm±	0.94	0.60	0.91	0.78

Culture period - Four weeks
Values taken as average of three replications
*MS medium containing full concentration of both inorganic salts and organic growth factors

33 days for culture elongation. Treatment combination involving NAA 2 ppm + BA 7.5 ppm registered minimum number of days for elongation in shoot tip explants. For eye bud explants, treatment combination involving NAA 2 ppm + BA 5 ppm recorded minimum number of days for culture elongation, and this was on par with the treatment NAA 2 ppm + BA 7.5 ppm.

4.3.1.2 Effect of KIN and NAA

Establishment of cultures occurred in all the eight combinations of NAA and KIN tried (Table 7). Shoot tip explants took 11 to 23.33 days and eye bud explant 12.66 to 26 days for culture establishment.

Treatment combination involving KIN 10 ppm + NAA 2 ppm was significantly superior to all other treatments. It was observed that with this combination, shoot tip and eye bud explants took minimum number of days only (11 and 12.66 respectively) for culture establishment. Treatment combination involving NAA 1 ppm + KIN 2.5 ppm recorded the maximum number of days (23.33 and 26 respectively) for culture establishment of the shoot tip and eye bud explants.

For culture elongation, shoot tip explant took 17.66 to 30 days and eye bud explants 19.66 to 32.67 days. Treatment combination involving NAA 2 ppm + KIN 10 ppm was significantly superior to all other treatments for culture elongation. It was observed that this treatment combination registered the minimum number of days

Table 7. Effect of NAA + Kinetin on the establishment of culture of shoot tip and eye bud explants of Musa (AAB) 'Nendran'

Basal medium - MS[a*]

Treatments	Shoot tip		Eye bud	
	Time taken for culture establishment (days)	Time taken for culture elongation (days)	Time taken for culture establishment (days)	Time taken for culture elongation (days)
NAA 1 ppm + KIN 2.5 ppm	23.33	30.00	26.00	32.67
NAA 1 ppm + KIN 5 ppm	20.00	27.33	21.66	29.00
NAA 1 ppm + KIN 7.5 ppm	21.33	29.33	23.00	29.66
NAA 1 ppm + KIN 10 ppm	17.33	23.66	21.00	28.66
NAA 2 ppm + KIN 2.5 ppm	16.33	22.66	18.00	25.33
NAA 2 ppm + KIN 5 ppm	14.33	20.33	16.33	23.33
NAA 2 ppm + KIN 7.5 ppm	14.00	21.00	16.66	23.33
NAA 2 ppm + KIN 10 ppm	11.00	17.66	12.66	19.66
CD (0.05)	1.97	2.51	2.03	2.03
SEm±	0.66	0.84	0.68	0.68

Culture period - Four weeks
Average of three replications
*MS medium containing full concentration of inorganic salts and organic growth factors

(17.66 and 19.66 respectively) for culture elongation of the shoot tip and eye bud explants. Maximum number of days for culture elongation was registered by the treatment combination involving NAA 1 ppm + KIN 2.5 ppm for both shoot tip and eye bud explants.

In Musa (AAB) 'Nendran' among the different concentrations of growth regulators tried for culture establishment and elongation, the treatment involving NAA 2 ppm + BA 5 ppm registered the minimum number of days (8.33 and 14.33 respectively) for culture establishment of the shoot tip and eye bud explants. Also it was observed that the treatment combination involving NAA 2 ppm + BA 7.5 ppm registered the minimum number of days (16.33) for culture elongation of shoot tip explants and for eye bud explants, the treatment combination involving NAA ppm + KIN 10 ppm recorded the minimum number of days (19.6 for culture elongation. The shoot tip explants which had sho earlier response to culture establishment than that of eye bud lants, showed earlier culture elongation as well in both the trials ducted for culture establish ment of Musa (AAB) Nendran.

4.3.2 Culture proliferation (Stage 2)

The results of the detailed onducted t modify the establishment medium and to arrive al proliferation medium for the induction and growth of hoots using different combinations of auxin (NAA) and c in MS^a and MS^b

media and the use of BA alone in the same media using different explants namely shoot tips and eye buds are presented in the Table 8.

4.3.2.1 Shoot tip

There was no significant difference among the treatments tried with regard to percentage of cultures developing shoots. The percentage of culture developing shoots were maximum (78.33) in the treatment combination involving MS^a + NAA 1 ppm + BA 5 ppm, MS^a + NAA 1 ppm + BA 10 ppm and MS^b + NAA 2 ppm + BA 5 ppm. All the treatment combinations developed multiple shoots, the number ranging from 1.33 to 8.66. The maximum number of shoots (8.66) was produced by the treatment involving MS^b + NAA 2 ppm + BA 10 ppm which was on par with MS^b + NAA 1 ppm + BA 10 ppm, MS^a + NAA 2 ppm + BA 7.5 ppm and MS^a + NAA 2 ppm + BA 10 ppm.

Hard greenish coloured globular callus like structures namely calloids, were formed in the treatment involving MS^a + BA 10 ppm. Regeneration of calloid was observed in the same media (Plate 1a).

4.3.2.2 Eye bud

The percentage of cultures developing shoots was maximum (80) at the treatment combination involving MS^b + NAA 2 ppm + BA 7.5 ppm. The number of shoots produced per culture ranged from 1 to 5. The maximum number of shoots was produced in treatment involving MS^a + NAA 2 ppm + BA 5 ppm. Calloid formation was not observed in any of the treatments.

Table 8. Effect of BA alone and in combination with NAA on culture proliferation of Musa (AAB) 'Nendran'

Basal medium - MS^{a*}

Treatments	Shoot tip		Eye bud	
	Culture developing shoots (%)	Shoots/ culture (No.)	Culture developing shoots (%)	Shoots/ culture (No.)
MS^{a*} + NAA 1 ppm + BA 5 ppm	78.33	5.00	53.33	3.00
MS^{b**} + NAA 1 ppm + BA 5 ppm	76.66	5.66	60.00	3.33
MS^{a} + NAA 1 ppm + BA 7.5 ppm	70.00	6.66	73.33	4.33
MS^{b} + NAA 1 ppm + BA 7.5 ppm	73.33	6.66	68.33	2.33
MS^{a} + NAA 1 ppm + BA 10 ppm	78.33	7.33	78.33	3.66
MS^{b} + NAA 1 ppm + BA 10 ppm	75.00	8.33	73.33	2.66
MS^{a} + NAA 2 ppm + BA 5 ppm	71.66	6.66	63.33	5.00
MS^{b} + NAA 2 ppm + BA 5 ppm	78.33	6.00	70.00	3.33
MS^{a} + NAA 2 ppm + BA 7.5 ppm	70.00	8.33	68.33	4.00
MS^{b} + NAA 2 ppm + BA 7.5 ppm	71.66	7.66	80.00	2.66
MS^{a} + NAA 2 ppm + BA 10 ppm	68.33	8.33	66.66	3.33
MS^{b} + NAA 2 ppm + BA 10 ppm	76.66	8.66	76.66	2.66
MS^{a} + BA 10 ppm	63.33	1.33	68.33	1.00
MS^{b} + BA 10 ppm	75.00	2.33	66.66	1.66
CD (0.05)	NS	2.3	14.19	1.56
SEm±	5.9	1.13	6.9	0.76

Culture period - Four weeks

Observation taken as average of three replications

*MS media containing full concentration of both inorganic salts and organic growth factors

** Ms media containing half concentration of inorganic salts

The results of the trial conducted so far indicated that shoot tips and eye buds could be chosen as suitable explants for rapid in vitro propagation of banana as they can be successfully used for the induction of numerous shoots within a short period. Of the 14 treatments tried using shoot tip and eye bud explants, it was observed that, shoot tip explants, produced maximum number of shoots (8.66) in the treatment involving MS^b + NAA 2 ppm + BA 10 ppm compared to eye bud explants which produced 5 shoots per culture in the treatment involving MS^a + NAA 2 ppm + BA 5 ppm.

4.4 Effect of continuous subculturing

Continuous subculturing was carried out at two week intervals to assess the variation induced to cultured plants due to repeated subculturing. During the culture period there was an increase or decrease in multiplication rate of axillary shoots as given in the Table 9. The plants developed from different subcultures are shown in the Plates 3, 4, 5, 6, 7, 8 and 9.

4.4.1 Shoots per culture

There was no significant difference among the subcultures tried with respect to the production of shoots per culture. Maximum number of shoots (7.4) was recorded at the 8th subculture followed by the 6th (6.6) and the 5th (6.4). Minimum number of shoots were produced (2.2) at the 1st subculture which was closely followed by the 2nd subculture (2.4).

Table 9. Influence of subculturing at 2 week interval on multiplication rate of shoot tip explants from the initial shoot proliferating cultures Musa (AAB) 'Nendran'

Basal medium - MS[a**]

No. of culture	Shoots/culture*	*Increase in number of shoots over the initial culture (%)
1	2.2	0
2	2.4	16.88
3	3.4	36.00
4	3.2	14.28
5	6.4	52.38
6	6.6	53.49
7	2.8	3.70
8	7.4	57.44
9	3.2	23.00
10	3.4	35.99
CD (0.05)	NS	
SEm±	8.3	

Total culture period - 18 weeks

* Average of five replications

**MS media containing full concentration of both inorganic salts and organic growth factors

4.4.2 Percentage increase in number of shoots

The percentage increase in number of shoots over the initial culture was estimated and the maximum value (57.44) was obtained at the 8th subculture followed by the 6th (53.49) and the 5th (52.38) subcultures.

4.5 Proliferation of calloid

Regenerated calloids (Plate 1b) from the media were subcultured into different combinations of auxin and cytokinins (Table 10) and it was observed that all the six treatments registered cent per cent culture proliferation (Plate 2). The maximum number of shoots (4) per culture was produced on MS^a + KIN 5 ppm + NAA 1 ppm and on MS^b + KIN 5 ppm + NAA 1 ppm. The minimum number (2.25) of shoots per culture was registered by the treatment combination involving basal MS^b media.

4.6 *In vitro* rooting and acclimatization (Stage 3)

The details of the experiment on *in vitro* rooting are presented in the Table 11. There was no significant difference between the media (MS media with full concentration of both inorganic salts and organic growth factors and MS media with half concentration of organic growth factors) in the rooting of banana shoots cultured *in vitro*. Among the six treatment combinations tried with different levels of NAA for rooting of banana shoots *in vitro*,

Table 10. Effect of different combinations of auxin and cytokinin on calloid regeneration

Treatments	Number of buds/ culture	Percentage of culture producing buds
MS^{a*} + BA 5 ppm + NAA 1 ppm	2.75	100
MS^{a} + KIN 5 ppm + NAA 1 ppm	4.00	100
MS^{a} + basal	2.50	100
MS^{b**} + BA 5 ppm + NAA 1 ppm	3.20	100
MS^{b} + KIN 5 ppm + NAA 1 ppm	4.00	100
MS^{b} + basal	2.25	100

Culture period - Four weeks

Observations are taken as mean of four cultures

* Murashige and Skoog media with full concentration of both inorganic salts and organic growth factors

** Mursahige and Skoog media with half concentration of organic growth factors

Table 11. Effect of NAA + Activated charcoal on the rooting of banana shoots Musa (AAB) 'Nendran'

Treatments	Root initiation (%)	Days taken for root initiation	Roots/shoot (No.)
MS^{a*} + NAA 2 ppm + AC 0.05%	100	4.67	3.33
MS^{b**} + NAA 2 ppm + AC 0.05%	100	6.00	4.66
MS^{a} + NAA 5 ppm + AC 0.05%	100	14.67	5.67
MS^{b} + NAA 5 ppm + AC 0.05%	100	15.33	3.67
MS^{a} + NAA 10 ppm + AC 0.05%	100	6.33	4.33
MS^{b} + NAA 10 ppm + AC 0.05%	100	4.00	2.67
CD (0.05)		1.81	NS
SEm±		0.59	0.74

Culture period - Four weeks

Values taken as average of three replications

*MS medium containing full concentration of both inorganic salts and organic growth factors

**MS medium containing half concentration of inorganic salts and full concentration of organic growth factors

Plate 1a. Formation of calloids and their regeneration observed from shoot tip culture of Musa (AAB) 'Nendran'

Plate 1b. Regenerated calloids

1a

1b

Plate 6. Rooted shoot from 5th subculture

Plate 7. Rooted shoot from 6th and 7th subculture

Plate 2. Proliferation of calloid Musa (AAB) 'Nendran'

Plate 3. Rooted shoot from 1st subculture

2

3

Plate 4. Rooted shoot from 2nd and 3rd subculture

Plate 5. Rooted shoot from 4th subculture

4

5

Plate 6. Rooted shoot from 5th subculture

Plate 7. Rooted shoot from 6th and 7th subculture

6

Plate 8. Rooted shoot from 8th and 9th subculture

Plate 9. Rooted shoot from 10th subculture

all the treatments registered cent per cent root initiation.

4.6.1 Days taken for root initiation

Among the treatments tried, treatment combination involving MS^b + NAA 10 ppm + Activated charcoal 0.05 per cent registered minimum number of days (4.00) for root initiation, which was on par with the treatment involving MS^a + NAA 2 ppm + AC 0.05 per cent (4.67 days). The maximum number of days (15.33) taken for root initiation was recorded by the treatment involving MS^b + NAA 5 ppm + AC 0.05 per cent. Therefore, it was concluded that, treatments like MS^b + NAA 10 ppm + AC 0.05 per cent and MS^a + NAA 2 ppm + AC 0.05 per cent were significantly superior to the other treatments tried.

4.6.2 Roots per shoot

There was no significant effect of any of the treatments tried on the number of roots produced per shoot. The number of roots produced per shoot ranged from 2.67 to 5.67.

In general, it was observed that the treatment (MS^b + NAA 10 ppm + AC 0.05%) which recorded minimum days (4.00) for root initiation produced lesser number of roots (2.67 roots per shoot). The maximum (5.67) roots per culture was produced by the treatment involving MS^a + NAA 5 ppm + AC 0.05 per cent, which had taken 14.67 days for culture initiation. Hence it can be concluded that,

the best treatment is that involving MS^b + NAA 2 ppm + AC 0.05 per cent, which recorded 6.00 days for root initiation and the number of roots obtained per shoot was 4.66. So the same treatment (MS^b + NAA 2 ppm + AC 0.05%) was used for rooting of proliferated calloid shoots also. Rooting occured after sixteen days of culturing. The number of roots produced per shoot varied from 6 to 8 in all the cultures.

4.7 Somatic organogenesis

4.7.1 Culture establishment (Stage 1)

4.7.1.1 Effect of different media

The data generated from the trial conducted to assess the effect of different media on callus initiation are presented in the Table 12.

4.7.1.1.1 Effect of MS^a media

Maximum survival per cent (90.48 and 85.70 respectively) for scalp and embryonic leaf explants were recorded at the treatment involving MS^a media of liquid consistency kept under dark condition. The minimum time (10.66) taken for callus initiation from scalp and embryonic leaf explants was also recorded for the same treatment. The same treatment also registered the maximum percentage (67.72) of cultures initiating callus in the case of embryonic leaf explants. For scalp explants, the maximum percentage (75.00) of cultures initiating callus was recorded for the treatment involving MS^a media of semi-solid consistency, kept under dark condition.

Table 12. Effect of different media on culture establishment of Musa (AAB) 'Nendran'

Culture media - Media + 2,4-D 5 ppm + BA 1 ppm

Media	Media consistency	Explant	Survival (%)		Time taken for callus initiation		Culture initiating callus (%)	
			Light*	Dark*	Light*	Dark*	Light*	Dark*
MS[a]**	Semi-solid	Embryonic leaves	Nil	Nil	Nil	Nil	Nil	Nil
	Liquid		Nil	85.70	Nil	10.66	Nil	67.72
	Semi-solid	Scalp from shoot tip	10.6	40.00	14.00	12.00	50.00	75.00
	Liquid		Nil	90.48	Nil	10.66	Nil	57.13
MS[b]***	Semi-solid	Embryonic leaves	Nil	33.33	Nil	14.00	Nil	50.00
	Liquid		Nil	96.48	Nil	9.66	Nil	98.66
	Semi-solid	Scalp from shoot tip	20.00	40.50	15.00	13.00	40.00	40.00
	Liquid		50.00	98.83	17.00	7.33	60.00	77.22
SH	Semi-solid	Embryonic leaves	Nil	Nil	Nil	Nil	Nil	Nil
	Liquid		Nil	36.83	Nil	23.16	Nil	31.66
	Semi-solid	Scalp from shoot tip	Nil	Nil	Nil	Nil	Nil	Nil
	Liquid		Nil	46.66	Nil	24.00	Nil	30.83
LS	Semi-solid	Embryonic leaves	Nil	Nil	Nil	Nil	Nil	Nil
	Liquid		Nil	41.66	Nil	23.33	Nil	28.33
	Semi-solid	Scalp from shoot tip	Nil	Nil	Nil	Nil	Nil	Nil
	Liquid		23.33	46.66	29.00	28.66	33.00	31.66

Values were taken as mean of three observations

Culture period - Four weeks

* Culture condition

** MS medium containing full concentration of both inorganic salts and organic growth factors

*** MS medium containing half concentration of inorganic salts and full concentration of organic growth factors

4.7.1.1.2 Effect of MS^b media

Maximum survival per cent (98.83 and 96.48 respectively) for scalp and embryonic leaf explants were recorded for the treatment involving MS^b media of liquid consistency and which were kept under dark condition. The same treatment recorded the minimum time for callus initiation (7.33 and 9.66 respectively) and maximum percentage of (77.22 and 98.66 respectively) cultures initiating callus with respect to scalp and embryonic leaf explants.

4.7.1.1.3 Effect of SH media

The treatment involving SH media of liquid consistency, kept under dark condition recorded survival percentages of 46.66 and 36.83 respectively for scalp and embryonic leaf explants. Callus initiation recorded in 24 and 23.16 days respectively while the percentage of culture initiating callus was 30.83 and 31.66 for scalp and embryonic leaf explants respectively. No other treatment in this media showed any response.

4.7.1.1.4 Effect of LS media

For embryonic leaves, the treatment involving LS media of liquid consistency, kept under dark condition recorded 41.66 per cent culture survival and 28.33 per cent of cultures initiating callus. While callus initiation occured within a mean number of 23.33 days. For scalp explants maximum survival per cent (46.66) was recorded for the treatment involving LS media of liquid consistency kept under dark condition. The same treatment recorded the minimum

number of days for callus initiation. The treatment involving LS media of liquid consistency kept under illuminated condition registered the maximum percentage (33) of cultures initiating callus.

Among the media tried for callus initiation with different explants, MS^b media recorded maximum survival for scalp and embryonic leaf explants (98.83 and 96.48 respectively) as well as the minimum time for callus initiation (7.33 and 9.66 respectively) and the maximum percentage of cultures initiating callus (77.22 and 98.66 respectively) for scalp and embryonic leaf explants. From the above trial it was also observed that liquid consistency of the medium and the dark culture condition were favourable for culture establishment of banana.

4.7.1.2 Effect of growth regulators

Data generated from the trial conducted to assess the effect of auxins and cytokinins on the culture establishment of shoot tip, embryonic leaf and scalp explants is presented in Table 14. The media used was MS^b, which was identified as the best media from the previous trial conducted to assess the effect of media on culture establishment.

4.7.1.2.1 Survival per cent

Among various treatments tried, the treatment involving 2,4-D 5 ppm and BA 1 ppm registered the maximum percentage of culture

Table 14. Effect of different combinations of auxins and cytokinin on the culture establishment of various explants of banana Musa (AAB) 'Nendran'

Medium - MS[b**]

Explant	Treatments	Culture establishment		
		Survival* (%)	Culture* initiating callus (%)	Days taken* for callus initiation
Shoot tip	2,4-D 5 ppm + BA 1 ppm	44.73	23.33	27.00
	2,4-D 7 ppm + BA 1 ppm	27.83	13.80	31.33
	2,4,5-T 5 ppm + BA 1 ppm	36.43	20.96	34.66
Embryonic leaves	2,4-D 5 ppm + BA 1 ppm	96.46	98.66	9.66
	2,4-D 7 ppm + BA 1 ppm	98.16	62.50	10.66
	2,4,5-T 5 ppm + BA 1 ppm	96.26	69.98	9.33
Scalp from shoot tip (in vitro)	2,4-D 5 ppm + BA 1 ppm	98.66	77.22	7.33
	2,4-D 7 ppm + BA 1 ppm	75.06	98.16	7.33
	2,4,5-T 5 ppm + BA 1 ppm	80.40	96.33	8.33
CD (0.05)		9.29	2.72	2.72
SEm±		3.13	0.91	0.91

Culture period - Four weeks
Culture condition - Dark

* Observation taken as average of three replications

** MS medium containing half concentration of inorganic salts and full concentration of organic growth factors

survival (44.73 and 98.66 respectively) in the case of shoot tip and scalp explants. The same treatment was on par with the treatments involving 2,4-D 7 ppm + BA 1 ppm (75.06 per cent) for scalp explants and 2,4,5-T 5 ppm + BA 1 ppm (80.40 and 36.43 per cent respectively) for scalp and shoot tip explants. For embryonic leaves the maximum survival percentage (98.16) was recorded for the treatment involving 2,4-D 7 ppm + BA 1 ppm.

4.7.1.2.2 Cultures initiating callus

All the treatments tried had a significant effect on the different explants with respect to callus initiation. The maximum percentage of cultures initiating callus was recorded for the treatment involving 2,4-D 5 ppm + BA 1 ppm for shoot tip and embryonic leaf explants (23.33 and 98.66 respectively). With 2,4-D 7 ppm + BA 1 ppm, a percentage of 98.16 of cultures initiating callus was obtained. for scalp explant

4.7.1.2.3 Time taken for callus initiation

Days taken for callus initiation varied from 27 to 34.66 days for shoot tip explants, 9.33 to 10.66 days for embyonic leaves and 7.33 to 8.33 days for scalp explants. Treatment involving 2,4-D 5 ppm + BA 1 ppm recorded the minimum number of days (27.00) for culture establishment of shoot tip explants. For embryonic leaves, the treatment 2,4,5-T 5 ppm + BA 1 ppm registered the minimum number of days (9.33) for culture establishment. Treatments like 2,4-D

5 ppm + BA 1 ppm and 2,4-D 7 ppm + BA 1 ppm recorded minimum days (7.33) for culture establishment of scalp explants.

4.7.1.3 Effect of explant

The response of various explants of banana var. Nendran to the treatments tried for callus initiation are summarised in the Table 13.

Among the various explants tried, embryonic leaf (Plate 10) and scalp explants (Plate 11) recorded maximum callusing. Inflorescence axis and flower base explants did not show any response to various treatments for callus initiation. Embryonic leaves recorded maximum callusing in the treatment combinations of 2,4-D 7 ppm + BA 1 ppm and 2,4,5-T 5 ppm + BA 1 ppm. Scalp explants recorded maximum callusing at the treatments involving 2,4-D 5 ppm + BA 1 ppm and 2,4,5-T 5 ppm + BA 1 ppm. Low levels of callusing were recorded in all the treatments in the case of shoot tip explants (Plate 12).

4.7.2 Culture proliferation (Stage 2)

The results of the detailed trials conducted to modify the establishment medium and to arrive at a suitable basal proliferation medium for callus growth using different combinations of auxins (2,4-D and 2,4,5-T) and cytokinin (BA) in MS (semi solid) medium using different explants namely, shoot tip, embryonic leaves and scalp explants are presented in Table 15.

Table 13. Response of various explants of banana Musa (AAB) 'Nendran' on callus initiation

Medium - MS[b**]

Explants	Callus initiation		
	2,4-D 5 ppm* + BA 1 ppm	2,4-D 7 ppm* + BA 1 ppm	2,4,5-T 5 ppm* + BA 1 ppm
Shoot tip	+	+	+
Embryonic leaves	+	+++	+++
Inflorescence axis	Nil	Nil	Nil
Flower base	Nil	Nil	Nil
Scalp from shoot tip (in vitro)	+++	++	+++

(+) Low level of callusing
(++) Medium callusing
(+++) Callus has covered the explant

Culture condition - Dark
* Average of 10 observations
Culture period - Four weeks

** MS medium containing half concentration of inorganic salts and organic growth factors

Table 15. Effect of different combinations of auxins and cytokinins on the culture proliferation of callus Musa (AAB) 'Nendran'

Treatments	Shoot tip			Embryonic leaves			Scalp from shoot tip		
	Culture initiating callus (%)	Growth score (G)	Callus index (CI)	Culture initiating callus (%)	Growth score (G)	Callus index (CI)	Culture initiating callus (%)	Growth score (G)	Callus index (CI)
MS[b]** +2,4-D 5 ppm+BA 1 ppm	23.30	2.00	46.60	98.66	1.66	163.77	77.22	2.50	193.05
MS[b]+2,4-D 7 ppm+BA 1 ppm	13.80	1.37	19.01	62.50	1.91	119.79	98.16	2.87	181.71
MS[b]+2,4,5-T 5 ppm+BA 1 ppm	20.99	1.00	20.99	70.00	1.41	99.15	96.33	2.31	222.76
MS[a]* +2,4-D 1 ppm	43.33	1.50	65.00	38.33	0.42	13.88	15.00	1.66	24.90
MS[b]+2,4-D 1 ppm	-	-	-	-	-	-	76.66	2.00	153.32
MS[a]+2,4-D 2 ppm	66.66	1.50	83.32	50.00	2.00	100.00	100.00	2.66	266.00
MS[b]+2,4-D 2 ppm	-	-	-	100.00	2.16	216.60	100.00	2.02	201.60
MS[a]+2,4,5-T 2 ppm	30.15	1.25	37.68	83.33	1.87	156.24	78.33	1.66	130.55
MS[a]+2,4,5-T 2.5 ppm	-	-	-	66.66	1.46	94.43	76.66	1.86	140.54
MS[a]+2,4-D 2 ppm+2,4,5-T 2 ppm	-	-	-	75.00	1.75	131.25	100.00	1.56	166.66
MS[b]+2,4-D 2 ppm+2,4,5-T 2 ppm	-	-	-	68.33	2.00	136.66	100.00	1.66	158.33

Culture period - Four weeks

* Values taken as average of ten observations

** MS medium containing full concentration of both inorganic salts and organic growth factors

** MS medium containing half concentration of inorganic salts and full concentration of organic growth factors

Plate 10. Callus induction from embryonic leaf of Musa (AAB) 'Nendran'

Plate 11. Callus induction from scalp of Musa (AAB) 'Nendran'

Plate 10. Callus induction from embryonic leaf of Musa (AAB) 'Nendran'

Plate 11. Callus induction from scalp of Musa (AAB) 'Nendran'

10

11

Plate 12. Callus induction from shoot tip of Musa (AAB) 'Nendran'

Plate 13. Rhizogenesis from callused shoot tip explant

12

13

14

15

4.7.2.1 Shoot tip

Maximum callus initiation (66.66 per cent) was observed with the treatment combination involving MS^a + 2,4-D 2 ppm. Minimum callus initiation (13.80 per cent) occured with the treatment combination involving MS^b + 2,4-D 7 ppm + BA 1 ppm. It was found that most of the treatment involving 2,4-D alone showed maximum callusing (2,4-D 1 ppm - 43.33 per cent, 2,4-D 2 ppm - 66.66 per cent). Among the media tried, MS^a was found more effective (66.66 per cent) than MS^b (13.80 per cent) for callus induction.

The callus growth score recorded varied from 1.25 to 2.00. Among the treatments tried maximum growth score (2.00) was recorded for the treatment combination involving MS^b + 2,4-D 5 ppm + BA 1 ppm.

Callus index varied from 19.01 to 83.32. Maximum callus index (83.32) was recorded for the treatment involving MS^a + 2,4-D 2 ppm.

4.7.2.2 Embryonic leaves

Among the media tried, maximum callusing was observed in both the media (MS^a and MS^b) with 83.33 per cent and 98.66 per cent respectively. Maximum callus initiation (98.66 per cent) was observed for the treatment involving MS^b + 2,4-D 5 ppm + BA 1 ppm.

The growth score of callus was ranged from 1.42 to 2.16. Maximum growth score (2.16) was recorded for the treatment combination involving MS^b + 2,4-D 2 ppm, and minimum growth score (0.42) was recorded for MS^a + 2,4-D 1 ppm. None of the treatments showed any significant effect on the growth score.

The callus index varied from 13.88 to 216.60. The maximum callus index (216.6) was produced for the treatment combination involving MS^b + 2,4-D 2 ppm and the minimum callus index (13.88) for the treatment with MS^a + 2,4-D 1 ppm.

4.7.2.3 Scalp from shoot tip

The percentage of culture initiating callus ranged from 15 to 100 per cent. Cent per cent callus initiation was observed for the treatment involving MS^a + 2,4-D 2 ppm, MS^b + 2,4-D 2 ppm, MS^a + 2,4-D 2 ppm + 2,4,5-T 2 ppm and MS^b + 2,4-D 2 ppm + 2,4,5-T 2 ppm. Minimum callus initiation (15 per cent) was recorded for the treatment involving MS^a + 2,4-D 1 ppm.

The growth score recorded ranged from 1.56 to 2.87. The maximum growth score (2.87) was recorded for the treatment combination involving MS^b + 2,4-D 7 ppm + BA 1 ppm, and the minimum growth score (1.56) was recorded for the treatment combination involving MS^a + 2,4-D 2 ppm + 2,4,5-T 2 ppm.

The callus index ranged from 24.90 to 266. The maximum callus index (266.0) was recorded for the treatment combination involving MS^a + 2,4-D 2 ppm and the minimum callus index (24) was observed for the treatment involving MS^a + 2,4-D 1 ppm (Fig. 3).

Among the three explants tried maximum callus index (266) was recorded by scalp explants followed by embryonic leaves (216).

4.7.3 Callus differentiation

The data generated from the trial conducted to observe the effect of different levels of auxin and cytokinin on differentiation of callus are presented in the Table 16.

4.7.3.1 Shoot tip

In MS^a media, rhizogenesis was recorded for the treatment involving basal media of both liquid and semi-solid consistency (Plate 13). Medium callusing was observed for the treatment involving BA 5 ppm both at liquid and semi-solid consistency. The callus produced was white and friable. Slight growth of the callus was observed for the treatment involving 2,4-D 1 ppm in liquid medium. The treatment involving 2,4-D 2 ppm induced formation of dark coloured globular callus, in both liquid and semi solid medium. The other treatments did not show any response. In MS^b media, rhizogenesis was induced by the treatments involving BA

Table 16. Effect of different levels of auxin and cytokinin on callus differentiation Musa (AAB) 'Nendran'

Culture condition - Light
Basal medium - MS
Culture period - Four weeks

Treatments	Growth response											
	MS^{a} **						MS^{b} ***					
	Callus taken from						Callus taken from					
	Shoot tip		Embryonic leaves		Scalp (in vitro)		Shoot tip		Embryonic leaves		Scalp (in vitro)	
	Liquid*	Semi-solid*	Liquid*	Semi-solid*	Liquid*	Semi-solid*	Liquid*	Semi-solid*	Liquid*	Semi-solid*	Liquid*	Semi-solid*
BA 5 ppm	++	++	+	W	++	–	–	R	+	–	–	CG
BA 10 ppm	–	–	–	W	–	–	–	–	–	–	–	+
2,4-D 1 ppm	+	–	+	+	++	+	G	–	+	–	–	–
2,4-D 2 ppm	G	G	G	–	e	–	–	–	+	–	G	–
2,4-D 5 ppm	–	–	–	–	–	–	–	–	–	–	–	–
MS basal	R	R	–	–	CG	–	–	W	–	–	+	+
BA 1 ppm + 2,4-D 5 ppm	–	–	–	W	–	W	R	R	W	W	W	W

* Media consistency (liquid and semi-solid)

(+) Low level of callusing
(++) Medium callusing
(G) Dark coloured globular callus
(CG) Cream coloured globular callus
(R) Root formation
(W) Watery callus
(e) Embryo like structure

** MS medium containing full concentration of both inorganic salts and organic growth factors
*** MS medium containing half concentration of inorganic salts and full concentration of organic growth factors

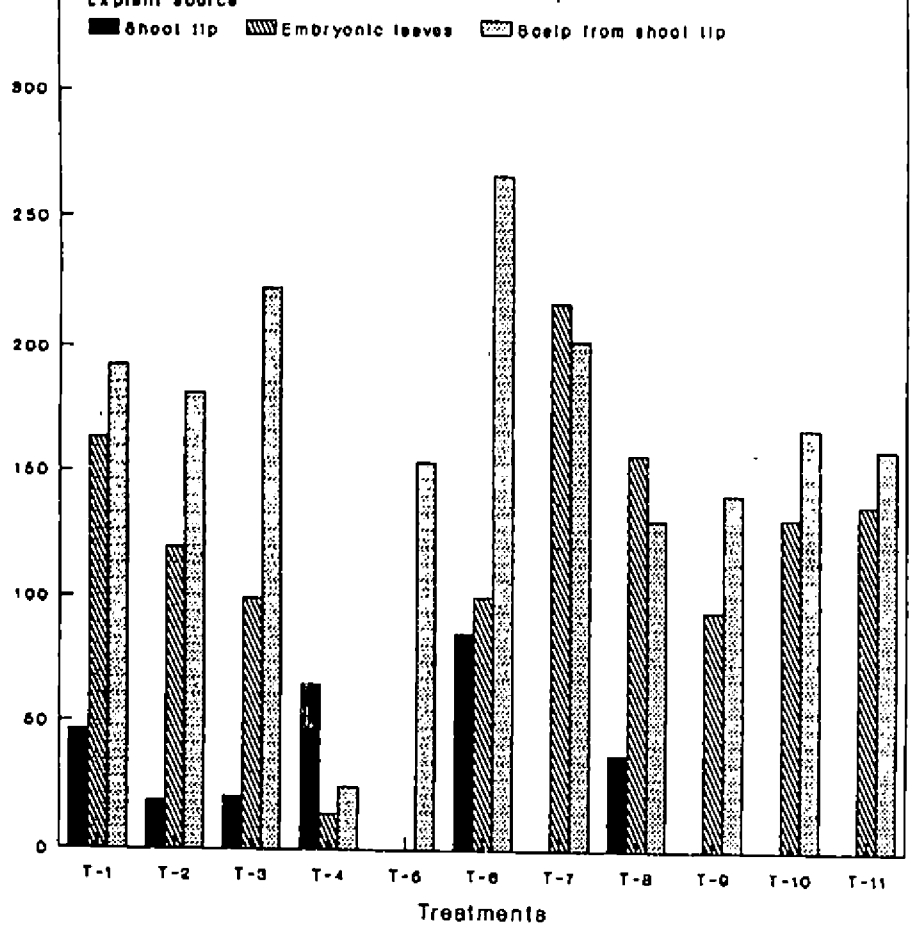


Fig.3 Effect of different combinations of auxins and cytokinins on the culture proliferation of callus (***Musa*** (AAB) 'Nendran').

T_1 - MS^{b**} + 2,4-D 5 ppm + BA 1 ppm
T_2 - " + 2,4-D 7 ppm + "
T_3 - " + 2,4,5-T 5 ppm + "
T_4 - MS^{a**} + 2,4-D 1 ppm
T_5 - MS^{b} + 2,4-D 1 ppm
T_6 - MS^{b} + 2,4-D 2 ppm
T_7 - MS^{b} + "
T_8 - MS^{a} + 2,4,5-T 2 ppm
T_9 - MS^{b} + 2,4,5-T 2.5 ppm
T_{10} - MS^{a} + 2,4-D 2 ppm + 2,4,5-T 2 ppm
T_{11} - MS^{b} + 2,4-D 2 ppm + 2,4,5-T 2 ppm

Murashige and Skoog media with full concentration of both in and organic growth factors

** Murashige and Skoog media with half concentration of organic factors

5 ppm (semi solid) and BA 1 ppm + 2,4-D 5 ppm, in liquid as well as semi solid medium. Dark coloured globular callus was also produced in the treatment with 2,4-D 1 ppm in liquid medium. Partially white and partially dark, watery callus was produced in the treatment involving basal semi solid media. The other treatments did not show any response.

4.7.3.2 Embryonic leaves

In MS^a media, slight growth of the cultured callus was observed for the treatment involving BA 5 ppm of liquid consistency and in 2,4-D 1 ppm of semi solid and liquid consistency. The callus produced was white and friable. Water soaked callus was induced with the treatment involving BA 5 ppm and BA 10 ppm of semi solid consistency and at a combination of BA 1 ppm + 2,4-D 5 ppm of semi solid consistency. In MS^b media, slight growth of the callus was observed for the treatments involving BA 5 ppm, 2,4-D 1 ppm and 2,4-D 2 ppm of liquid consistency. Watery callus growth was resulted with the combination involving BA 1 ppm + 2,4-D 5 ppm of liquid and semi solid medium (Plate 14).

4.7.3.3 Scalp

In MS^a media, medium growth of the callus was observed for the treatment combination involving BA 5 ppm and 2,4-D 1 ppm of liquid consistency. Cream coloured globular callus was formed in basal liquid media (Plate 15). Watery callus was observed for

Plate 14. Watery callus growth from callused embryonic leaf

Plate 15. Cream, globular callus growth observed on scalp callus

the treatment involving BA 1 ppm + 2,4-D 5 ppm. Treatment involving 2,4-D 1 ppm recorded only slight growth of the callus. Embryo like structures were observed in the treatment involving 2,4-D 2 ppm of liquid consistency. In MS^b media, cream coloured globular callus was produced in the treatment involving BA 5 ppm of semi solid consistency and in the treatment involving 2,4-D 2 ppm of liquid consistency, dark coloured golublar callus was observed. Low levels of callusing were observed by the treatments, involving BA 10 ppm and basal media of both semi solid and liquid consistency.

In general, the effect of different treatments on different banana explants, for callus differentiation indicated that, the treatment involving 2,4-D 2 ppm in liquid media (MS^a and MS^b) on callus obtained from scalp explants were more promising to callus differentiation.

4.7.4 Effect of nitrate source

The effect of increasing or decreasing the levels of the nitrate source in MS media was studied with different concentrations of growth regulators in another experiment. The results obtained are presented in Table 17.

4.7.4.1 Shoot tip

Use of the normal level of the nitrate source (NH_4NO_3 - 1.64 g; KNO_3 - 1.90 g) resulted in rhizogenesis in liquid MS media with

Table 17. Effect of different levels of Nitrate* source on callus differentiation

Basal media - MS^{a}**

Media consistency	Treatments	Shoot tip			Embryonic leaves			Scalp from shoot tip (in vitro)		
		Concentration of nitrate source in media			Concentration of nitrate source in media			Concentration of nitrate source in media		
		½*	1½*	Full*	½*	1½*	Full*	½*	Full*	1½*
MS^{a} (Semi-solid)	BA 5 ppm	R	–	++	+	+	W	+	–	G
	BA 10 ppm	–	–	–	+	–	W	+	–	–
	2,4-D 1 ppm	+	–	+	–	G	+	–	++	–
	2,4-D 2 ppm	R	G	G	G	–	–	–	e	–
	2,4-D 5 ppm	–	–	–	–	–	–	–	–	–
	Basal	+	–	++	W	+	–	W	–	–
MS^{a} (Liquid)	BA 5 ppm	–	–	R	+	–	+	+	++	–
	BA 10 ppm	+	–	–	+	–	–	–	–	–
	2,4-D 1 ppm	+	–	–	–	–	+	+	+	+
	2,4-D 2 ppm	–	+	G	–	–	G	+	–	+
	2,4-D 5 ppm	–	–	–	–	–	–	–	–	–
	Basal	–	–	R	–	–	–	–	CG	–

Values taken as average of five replications

Culture period - Four weeks

(+) Low level of callusing

(++) Medium callusing

(G) Dark coloured globular callus

(CG) Creamy globular callus

(R) Root formation

(W) Watery callus

(e) embryo like structure

**MS medium containing full concentration of both inorganic salts and organic growth factors

* Level of nitrate source - Normal (full) - 1900 mg/l KNO_3 and 1650 mg/l NH_4NO_3
½ - 950 mg/l " and 825 mg/l " } per one litre MS media
1½ - 2850 mg/l " and 2475 mg/l "

BA 5 ppm or basal media while with half the concentration of the nitrate source, rhizogenesis was observed in semi-solid media supplemented with BA 5 ppm or 2,4-D 2 ppm. With increased levels of the nitrate source no organogenesis was observed in any of the treatments.

Medium callus growth was observed with normal levels of the nitrate source in MS^a semi-solid basal as well as MS^a supplemented with BA 5 ppm. Low callus growth was observed when the nitrate source was reduced to half in semi-solid as well as liquid MS medium supplemented with 2,4-D 2 ppm or in liquid MS supplemented with BA 10 ppm and in basal semi-solid MS media. At increased level of the nitrate source formation of dark coloured globular callus was observed in semi-solid MS medium supplemented with 2,4-D 2 ppm while low levels of callusing occured in liquid MS medium with 2,4-D 2 ppm.

4.7.4.2 Embryonic leaves

Use of the normal level of nitrate source resulted in watery callus growth in semi-solid MS media with BA 5 ppm or BA 10 ppm. And formation of dark coloured globular callus was observed in liquid MS media with 2,4-D 2 ppm. The same growth was observed in MS semi-solid media modified with half the concentration of nitrate source and with 2,4-D 2 ppm. Also low level of callus growth was observed in semi-solid as well as liquid MS media supplemented

with BA 5 ppm and BA 10 ppm and watery callus were observed with basal semi-solid MS media. With increased level of nitrate source, formation of dark coloured globular callus was observed in semi-solid MS medium supplemented with 2,4-D 1 ppm while low level of callusing occured in basal semi-solid MS medium.

4.7.4.3 Scalp

Use of the normal level of nitrate source resulted in formation of embryoid like structures in semi-solid MS media and medium callusing in semi-solid and liquid media supplemented with 2,4-D 1 ppm and BA 5 ppm respectively. The same level of nitrate source also induced creamy globular callus with basal liquid MS media. At increased level of nitrate source dark coloured globular callus was observed with BA 5 ppm in semi-solid MS media and low level of callusing in MS liquid media with 2,4-D 1 and 2 ppm. Low level of callusing were observed when the nitrate source level was reduced and was supplemented with BA 5 ppm and BA 10 ppm in semi-solid MS media and BA 5 ppm, 2,4-D 1 and 2 ppm in liquid media. Also watery callus growth was observed with basal semi-solid MS media.

4.8 Somatic embryogenesis

From the trial conducted for callus induction and differentiation, it was observed that, the scalp explants were more amenable to callus induction and differentiation, within a shorter time as

well, compared to other banana explants used for the study. Hence the scalp was taken as the explant for induction of somatic embryogenesis (Table 18). Calli produced on the scalp explants were transferred to growth regulator free media and kept under light. After two weeks, whitish globular structures (Plate 16) were formed. However subculturing of these globular structures onto MS^a medium with BA 1 ppm resulted in no differentiation.

4.9 Planting out and hardening

When the plantlets developed 3-4 leaves and a good number of roots, they were taken out, washed under tap water to remove the residues of media, and were subjected to hardening treatment before transferring them into sand which involved, keeping the plantlets is distilled water for 8 h followed by 8 h in liquid MS medium (containing 1/10th strength of inorganic salts, with no sucrose) under microscope cover. After the hardening treatments, the roots of all the banana plantlets were dipped in 0.1 per cent Bavistin solution for five minutes. Then they were taken out, and before transferring to the pots, growth parameters of the plantlets from different subcultures were recorded. The data are presented in Table 19.

4.9.1 Plant height

Maximum plant height (12.50 cm) was recorded in plants from the 10th subculture which was followed by (11.20 cm) in the plants

Table 18. Somatic embryogenesis Musa (AAB) 'Nendran'

Explant	Treatment	Response
Scalp isolated from shoot tip (in vitro)	MS^b + BA 1 ppm + 2,4-D 5 ppm	Proliferating callus
Callus from scalp culture	Liquid basal medium	Embryo like globular structure

Culture period - Four weeks

Values taken as average of ten observations

Table 19. Growth characters in the rooted plantlets of Musa (AAB) 'Nendran' as influenced by subculturing

Culture number	Plant* height (cm)	Number of* leaves	Mean length* of the longest leaf (cm)	Number of* roots/ shoot	Mean length* of roots (cm)
1	4.80	2.0	3.0	5.30	4.60
2	6.36	2.3	3.5	5.00	2.80
3	6.13	2.6	3.5	6.66	12.80
4	4.70	2.0	4.4	4.66	11.56
5	6.80	2.3	4.5	7.00	9.16
6	11.20	3.0	4.3	5.33	13.00
7	9.30	2.3	6.0	4.33	13.00
8	10.00	3.0	5.5	5.66	2.50
9	9.70	2.6	6.0	5.00	3.48
10	12.50	3.0	6.0	4.33	6.67

Observations are taken at the time of transplanting

* Average of three observations were taken

Plate 16. Whitish embryo like growth on scalp callus

Plate 17. *In vitro* grown plantlets established in sand

16

17

from the 6th subculture. Minimum plant height (4.70 cm) was recorded by plants from 4th subculture.

4.9.2 Number of leaves

All the plants regenerated from all the subcultures produced more or less the same number of leaves.

4.9.3 Mean length of the longest leaf

Maximum length (6.0 cm) of the leaf was recorded by plants regenerating from 7th, 9th and 10th subculture, which was closely followed by plants originated from 8th subculture (5.5 cm). Minimum length of the leaf (3.0 cm) was recorded by plants regenerating from first subculture.

4.9.4 Number of roots/shoot

Maximum number of roots were produced (7 roots/shoot) from the plants regenerated from 5th subculture, which was closely followed (6.66) by 3rd subculture. Minimum number of roots/shoot (4.33) was produced by plants regenerating from 7th and 10th subcultures.

4.9.5 Mean length of the roots

Maximum root length (13.00 cm) was observed in the plants regenerating from 6th and 7th subcultures. Minimum length of the roots was recorded (2.50 cm) in the plants regenerating from 8th subculture.

4.10 Cytological studies

Somatic chromosome counts made at the in vitro root tips of plantlets from ten subcultures revealed that there was no variation in chromosome number due to subculturing. All the plantlets were triploids (2n = 33).

4.11 Evaluation of growth of plantlets

4.11.1 Effect of potting media on survival and growth of plantlets (Fig. 4a & 4b)

Different types of potting media were used for planting out (Table 20). Among the potting media, the most suitable was found to be sand. It recorded maximum survival of plantlets (90 per cent), which was closely followed by perlite (80 per cent). Biofibe registered minimum percentage of survival (10 per cent). Plants grown in sand registered maximum height (14.06 cm). Minimum height (4.6 cm) was registered by plants grown in soilrite. Number of leaf production in all the plants grown in the media under observation were same (2/3). Days taken for leaf production (17.5 days) was same for all the media except cocopeat (21.0 days).

4.11.2 Growth parameters

The plantlets from different subcultures were planted out in sand (Plate 17), which was found to be the best medium and observations were recorded on growth parameters at fifteen days interval to find out the variation if any, induced by subculturing.

Table 20. Effect of different potting media on the growth of *in vitro* raised plantlets of *Musa* (AAB) 'Nendran'

Potting media	Plant* survival (%)	Height* of the plant (cm)	Total number* of leaves	Average number of* days for leaf production
Sand	90	14.06	3.3	17.5
Perlite	80	12.36	3.6	17.5
Soilrite	75	4.6	2.6	17.5
Biofibe	10	11.36	2.6	17.5
Cocopeat	50	6.8	3.3	21.0

Growth period - Forty days

* Mean of four observations

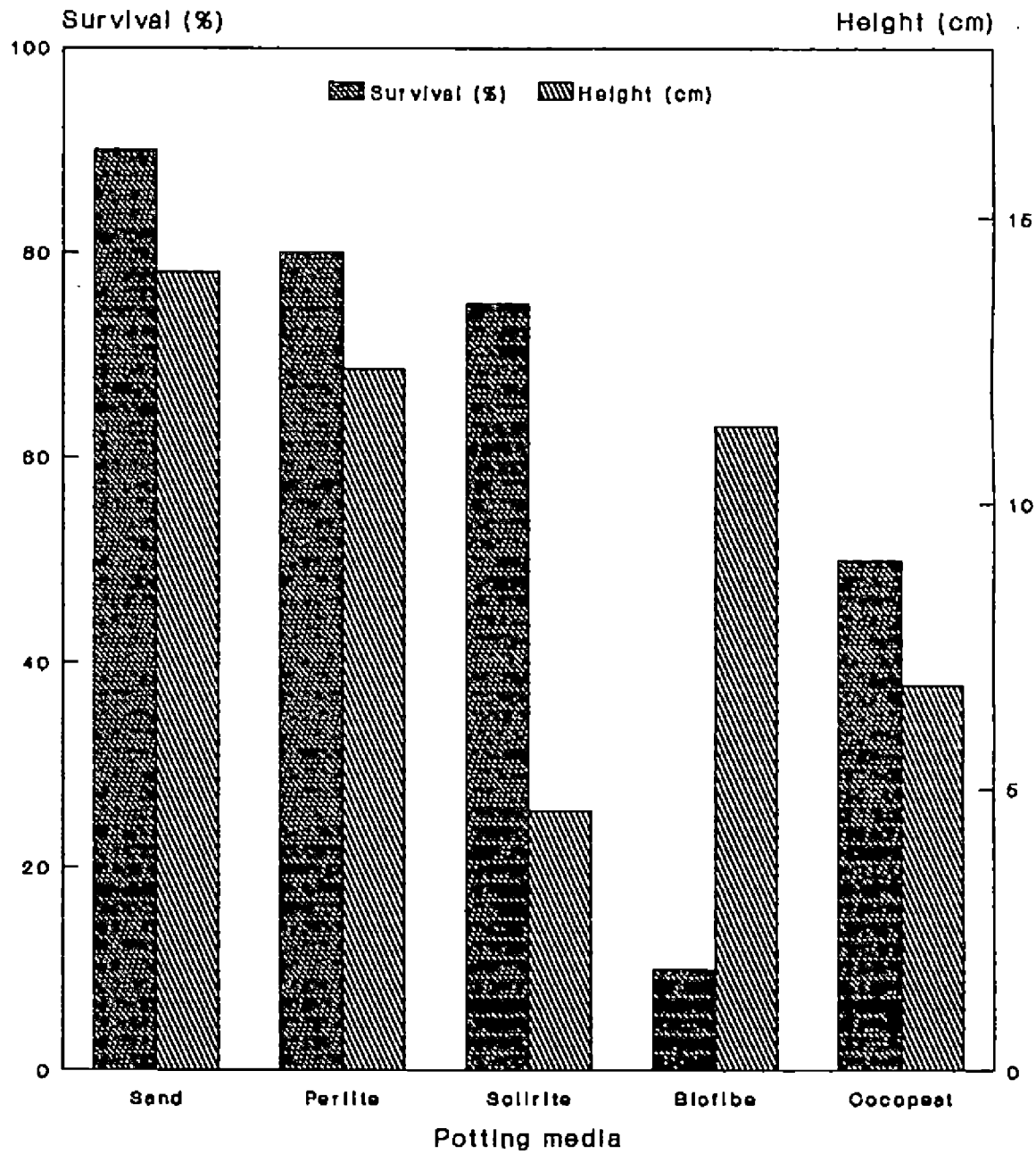


Fig. 4a. Effect of potting media on plant survival and height of ***In vitro*** plantlets of ***Musa*** (AAB) 'Nendran'.

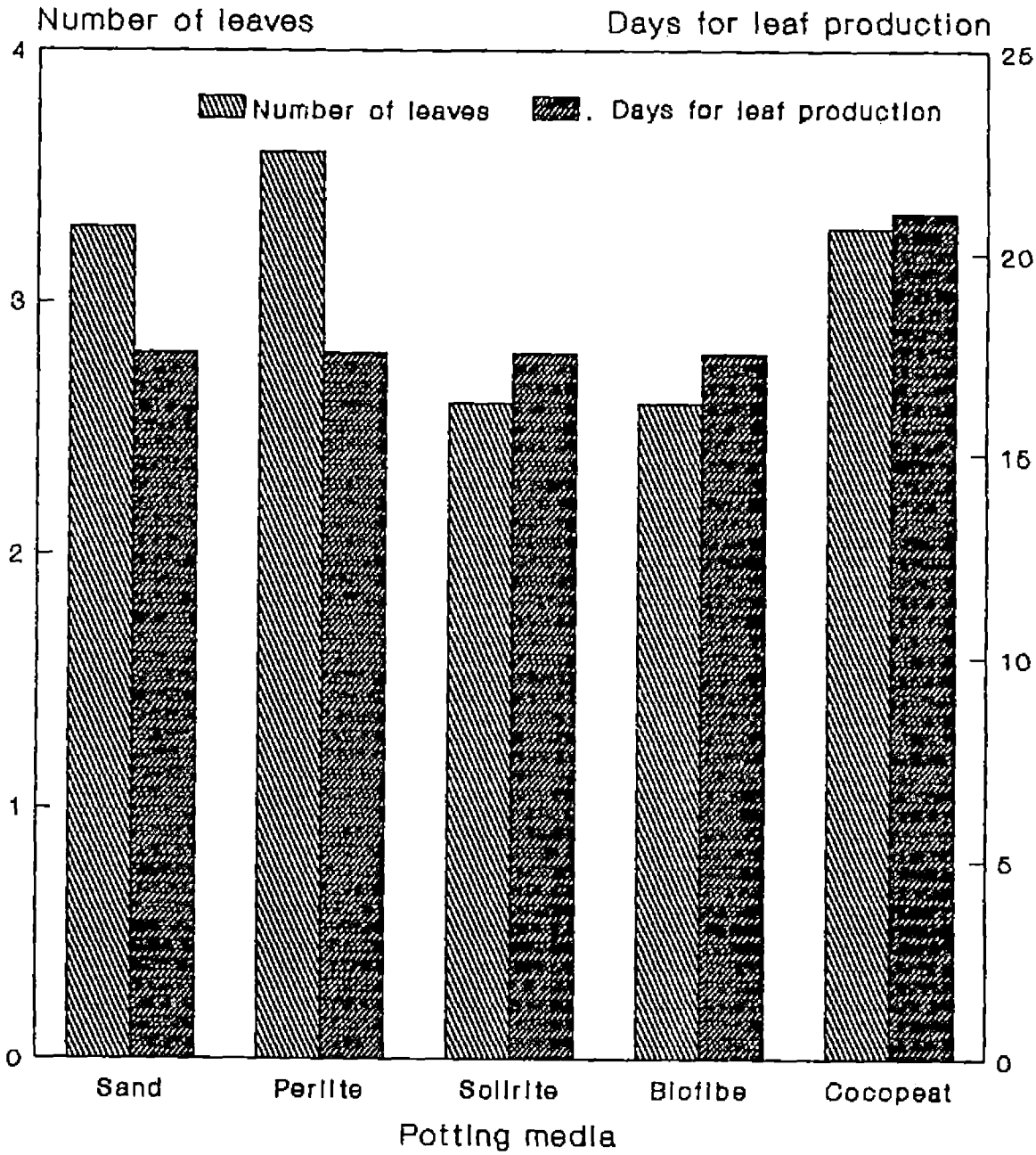


Fig.4b Effect of potting media on number of leaves and days for leaf production of ***In vitro*** plantlets of ***Musa*** (AAB) 'Nendran'.

Observations recorded sixty days after transplanting are presented in the Table 21.

4.11.2.1 Plant height

Maximum plant height (14.42 cm) was recorded at 10th subculture which was closely followed (13.95 cm) by 9th subculture. Minimum plant height (7.45 cm) was recorded at 4th subculture.

4.11.2.2 Girth of the plant

Maximum girth (2.87 cm) of the plant was recorded at 10th subculture and minimum girth (1.85) was recorded by 6th subculture.

4.11.2.3 Length of the leaf

Maximum length (8.50 cm) of the leaf was registered at 5th subculture which was closely followed (8.30 cm) by 4th subculture. Minimum length (4.30 cm) of the leaf was registered at 7th subculture.

4.11.2.4 Width of the leaf

Maximum width (2.73 cm) of the leaf was recorded at the 4th subculture and minimum width (1.18 cm) was recorded at 7th subculture.

4.11.2.5 Number of leaves

Maximum leaf production (3.25) was recorded by 9th subculture, almost all the plants at different subcultures produced 3.00

Table 21. Effect of subcultures on growth parameters of Musa (AAB) 'Nendran' sixty days after transplanting

Planting media - Sand

Culture	Plant height (cm)	Girth of the plant (cm)	Length of the* leaf (cm)	Width of the* leaf (cm)	Number of leaves (cm)
1	7.52	2.10	6.30	2.48	3.00
2	9.12	2.13	7.25	2.35	2.75
3	10.57	2.00	7.37	2.60	3.00
4	7.45	2.20	8.30	2.73	3.00
5	9.18	2.17	8.50	2.70	2.75
6	13.07	1.85	7.45	1.85	3.00
7	11.32	1.87	4.30	1.18	3.00
8	10.25	1.95	6.13	1.86	3.00
9	13.95	2.47	6.40	2.18	3.25
10	14.42	2.87	6.66	1.78	3.00
Mean					

Values taken as mean of four observations

Culture period - 8 weeks

* Observations were taken from the 3rd leaf

leaves. There was no wide variation with respect to number of leaves at different subcultures.

Difference in growth rate possessed by plantlets from different subcultures were calculated from the above observation (Table 22).

4.11.2.6 Growth rate of plants from different subcultures

4.11.2.6.1 Plant height

The growth rate of plantlets from different subcultures were calculated and it was seen that, plantlets from subcultures differed significantly in the rate of growth with respect to height. Maximum growth rate (1.03 cm) was recorded by 6th subculture which is on par with 2nd (0.88 cm) and 9th (0.84 cm) subcultures. Minimum growth rate (0.10 cm) was recorded by 4th subculture derived plants (Fig. 5).

4.11.2.6.2 Girth

There was no significant effect of subcultures in the rate of growth with respect to girth. However the maximum growth rate (0.53 cm) was recorded by fourth subculture.

4.11.2.6.3 Leaf area

Of the different subcultures tried, maximum growth rate with respect to leaf area (19.20 cm^2) was produced by 4th subculture. Minimum growth rate (4.07 m^2) was registered by 7th subculture.

Table 22. Effect of different subcultures on growth rate of Musa (AAB) 'Nendran'

Duration of observation - Two months

Culture	Mean growth rate (cm)		Leaf area* (cm^2)
	Height*	Girth*	
1	0.38	0.35	13.07
2	0.88	0.30	14.22
3	0.50	0.22	16.02
4	0.10	0.53	19.20
5	0.37	0.21	16.50
6	1.03	0.13	11.37
7	0.36	0.42	4.07
8	0.67	0.23	8.72
9	0.84	0.29	11.67
10	0.46	0.15	9.90
CD (0.05)	0.32	NS	
SEm±	0.15	0.23	

*Mean of four replication

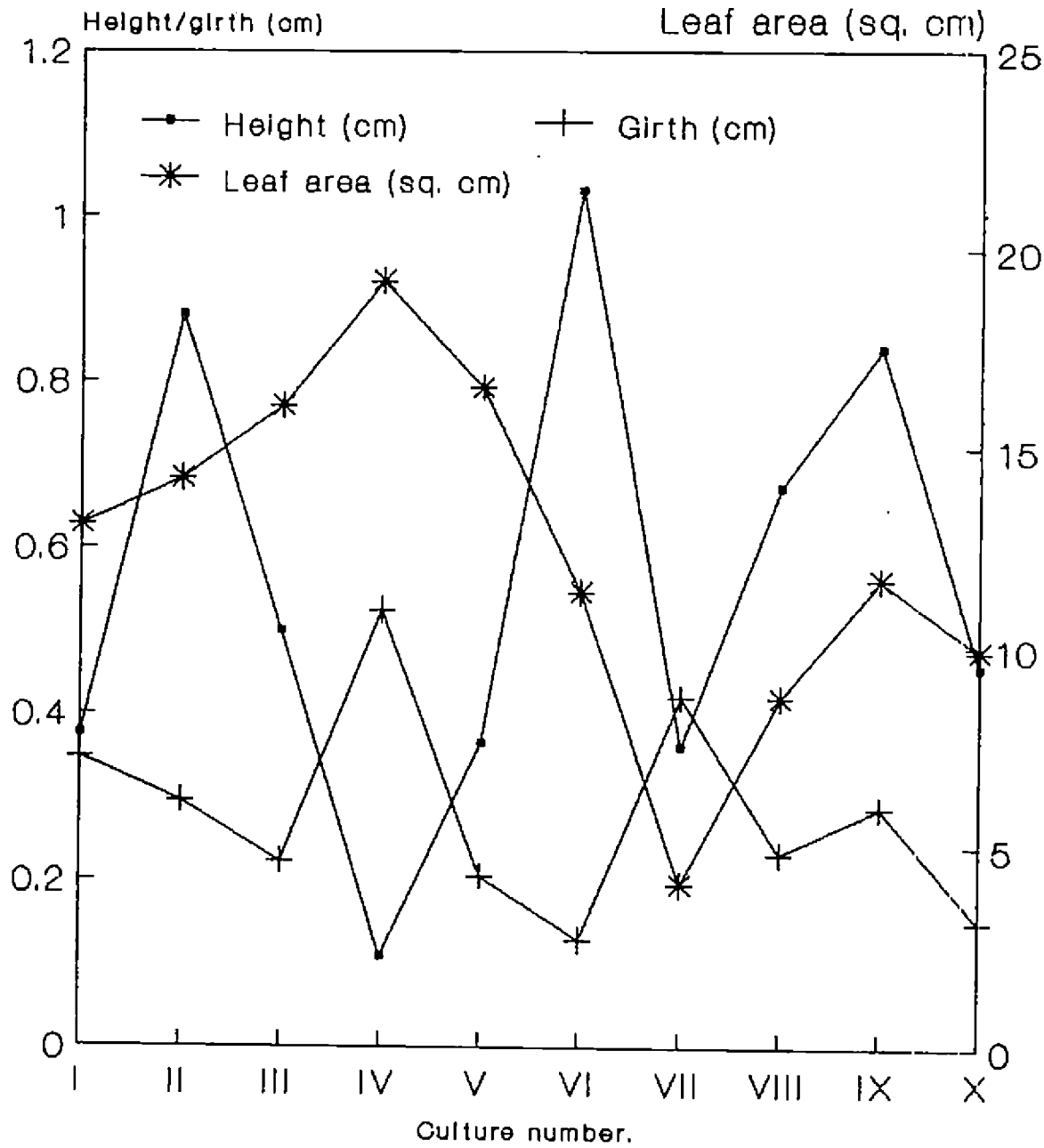


Fig.5. Effect of different subcultures on the Growth rate of ***Musa*** (AAB) 'Nendran'.

Discussion

)ISCUSSION

Banana is commonly propagated vegetatively through suckers. In recent years, aseptic culture techniques have been thought to hold great promise for addressing the various problems related to banana improvement. Micropropagation has many advantages like the rapid clonal multiplication of elite plants and year round production under controlled conditions, freedom from pests and diseases and amenability to various manipulations.

Tissue culture regeneration routed through callus, single cells or protoplasts, may be accompanied by minor or extensive changes in the final plant phenotype. This has been termed as somaclonal variation(Scowcroft et al., 1985).The probability of selecting potential useful mutations would be greately enhanced via cell culture techniques since many single cells could be affected and each of these would theoratically at least, be capable of growing cell cultures and can be grown in such a way as to produce variation certainly greater than that achievable by conventional breeding. It is also possible that continuous subculturing might result in somaclonal variation (Pierik, 1987). This has much importance in banana, where the natural variability is minimum because of continuous vegetative propagation. Moreover, the conventional hybridisation is also difficult in banana due to polyploidy and sterility, combined with the parthenocarpic development of fruits.

The present investigations carried out at the College of Horticulture, Vellanikkara were mainly aimed to study the methods to induce variation in banana by subjecting the explants of banana var. 'Nendran' to regenerate through a callus phase by somatic organogenesis/embryogenesis and through prolonged subculture cycles.

In micropropagation the organs and tissues are carried through a sequence of steps in which differential cultural and environmental conditions are provided. These steps have been indicated as different stages with stage I being physiological preconditioning of the explant and explant establishment, stage II rapid multiplication of shoots through increased axillary bud release, stage III rooting and hardening of *in vitro* raised plants and stage IV acclimatization and transplantation to field conditions (Murashige, 1974; Thorpe and Patel, 1984; Reghunath, 1989).

Banana being a monocotyledonous plant, never produces branches and the buds in the axils are dormant due to the 'apical dominance' shown by the fast growing meristematic tip. Stage I is to activate these buds to form branches by suppressing the apical dominance. Physical injury treatments like killing the bud apex was found to be favourable in the release of axillary buds. The technique of splitting banana shoot tips longitudinally through their apex in order to induce multiple shoot formation was first described by De Guzman *et al.* (1980) and then by Jarret *et al.* (1985).

5.1 Enhanced release of axillary buds

As the shoot tips and eye buds emerge from below the ground level, they accumulate lot of soil and dirt. This necessitates a thorough and effective surface sterilization of the explants before culturing. Of the various sterilants tried for shoot tip and eye bud explants an initial dipping of explants in Emisan (0.1 per cent) for 30 minutes followed by dipping in Norfloxacia (0.1 per cent) for 30 minutes and finally the rinsing of explants in mercuric chloride (0.1 per cent) for 20 minutes resulted in the least rate of contamination (11 and 20 per cent respectively) and maximum percentage of explant survival (88 and 80 respectively). For flower base and inflorescence axis explants the best sterilization treatment was found to be that using emisan (0.1 per cent) for 20 minutes which resulted in maximum percentage of survival (11 and 48.33 respectively). In the case of embryonic leaves dipping in alcohol for one second recorded maximum survival (80 per cent). Even though there are reports of mercuric chloride being more efficient when the explants are highly contaminated (Bhaskar, 1991), in the present study it was observed that a combination of different sterilants were more effective especially when explants were as highly contaminated, as the shoot tip and eye buds were emerged from below the ground level.

In banana, tissue and media discolouration was found to be a serious problem in establishing a good and healthy culture. This

discolouration resulted from the oxidation of polyphenols present in the explant tissues of banana. Several workers attempted various methods to overcome this problem but no method was found to result in complete success (Jarret et al., 1985; Vuylsteke and De Langhe, 1985 and Banerjee and De Langhe, 1985). Also it was reported that by addition of ascorbic acid into the media at the rate of 50 mg per litre, 90 per cent of the cultures were freed from this problem (Bhaskar, 1991). In the present study it was observed that, addition of activated charcoal into the media at the rate of 500 mg per litre reduced media and explant discolouration. The effect may be due to the properties of activated charcoal to adsorb inhibiting compounds and excessive concentration of plant growth hormones and thus preventing the oxidation of polyphenols, which resulted in the reduction of media and explant discolouration.

Jarret et al. (1985) reported that shoot tip cultures of two banana clones 'Saba' and 'Pelipita' established on a modified MS basal medium supplemented with IAA 1 ppm and BA 3 ppm. The results of the present study indicated that, for the better and speedier explant establishment and growth of shoot tip and eye bud explants, MS^a (semi-solid) medium containing NAA 2 ppm + BA 5 ppm, as well as NAA 2 ppm + Kinetin 10 ppm respectively was found to be the best. Compared to shoot tip explants (8.33 days), eye bud explants took more time (12.66 days) for culture establishment.

In shoot tip culture, on an average each explant released 8.66 axillary shoots, when MS^b containing NAA 2 ppm + BA 10 ppm was used. In the case of eye bud explant on an average each explant released 5 shoots in MS^a containing NAA 2 ppm + BA 5 ppm.

The favourable effects of axillary bud bursting and multiple shoot production by cytokinins had been demonstrated by Murashige (1974). But at higher levels, cytokinins were proved to have delterious effect on shoot growth. Auxin added to the medium helps to nullify the suppressive effect of high cytokinin concentration on axillary shoot growth (Lundergan and Janik, 1980). In the present study the same effect was recorded. Higher concentration of BA suppressed the axillary shoot production, but in most of the combinations involving NAA and BA, it was observed that auxin NAA favourably influenced cytokinin (BA) activity, when it was applied at higher concentration (10 ppm).

The basic phenomenon involved in the induction of axillary shoots and subsequent plantlet production in vitro are reported to be due to the action of plant hormones. Little is known about how hormones evoke a particular pattern of morphogenesis (Thorpe, 1980). One hypothesis is that hormone treatment starts the cells on a specific developmental pathway. Another view is that the hormone responsive cells are already determined and that hormones evoke the expression of the committed state. The available evidence suggests that the hormones act in both ways. Hence the observed

difference in organogenesis in the different explants from the same plant could be attributed to the differential requirement of growth hormones for each explant. According to Skoog and Miller (1957), quantitative interaction between diverse growth factors may have a decisive role in organogenesis. The shoot tip and eye bud explants took more time for culture establishment. This difference in performance of explants may be attributed to the difference in the level of endogenous phytohormones, nutrients and metabolites and the interaction between various growth factors. Drew et al. (1989) reported that sucker derived explants initiated shoots more readily than inflorescence derived explants. During the course of the study it was clearly understood that the cytokinins added in the medium play a prominent role in the induction of axillary shoots.

5.2 Prolonged subculture cycle

The effect of serial subculturing of an explant stock continuously for a long period of time was looked into. It was found that when subculturing was done at two week interval, the number of shoots produced per culture was not constant in all the subcultures. Still, the shoots produced per explant per culture vessal increased at a mean rate of 5.9. Increase in multiple shoot production continued from the Ist subculture onwards until 6th subculture, then there was a decline in the shoot production at 7th subculture, followed by a sudden increase in shoot production at 8th subculture. After the 8th subculture, there was a reduction in shoot production

in 9th and 10th subcultures. Several workers (Doreswamy *et al.*, 1983; Damasco *et al.*, 1984; Vuylsteke and De Langhe, 1985; Aravindakshan, 1989) had reported shoot multiplication rate achieved through serial subculturing in banana. It is supposed that continuous subculturing modifies the physiological state of the plant in such a way that it favour revitalisation of innate dormant vegetative buds (Litz and Conover, 1978; Franclet, 1979; David, 1982). Continuous subculturing was also found to reduce media and explant discolouration in banana *in vitro*.

5.3 Rooting

Stage 3 involves *in vitro* regeneration of roots from the shoots obtained from stage 2. Several researchers had shown that *in vitro* rooting can successfully be achieved by reducing salt concentration in the media particularly in high salt media like MS and its derivatives (Kartha *et al.*, 1974; Lane, 1979; Skirvin and Chu, 1979). Half concentration of MS medium was found to favourably induce the rooting of banana without affecting the shoot growth unlike in certain species (Wang, 1978; Gupta *et al.*, 1981). But in the present study, cent per cent root initiation was observed at half and full concentration of MS medium. Minimum days (4.00) for rooting was taken by the treatment involving MS^b containing NAA 10 ppm and AC 0.05 per cent. Maximum number of roots per shoot (5.67) was produced in the treatment involving MS^a containing NAA 5 mg + AC 0.05 per cent. Lane (1979) reported that NAA usually

gave rise to short thick roots. But in the present observation it was noted that, NAA at lower concentration (2 ppm and 5 ppm) produce long thin roots and NAA at higher concentrations (10 ppm) produced short thick roots. The favourable effects of AC in rooting had been reported by many workers (Wang and Huang, 1976; Banks and Hackett, 1979). The capacity to adsorb inhibiting compounds, and excessive concentration of plant growth hormones and the property to darken the medium which mimics the soil conditions are the factors proposed in favour of the favourable effects of AC in rooting (Proskauer and Berman, 1970; Wang and Huang, 1976; Fridborg et al., 1978). So in the present study, activated charcoal was a common factor in all the treatments tried.

5.4 Induction of calloids and regneration

Promising results were obtained with respect to calloid formation and subsequent plant regeneration.

When the shoot tip explants were kept for culture proliferation, greenish semihard globular structures viz. calloids, as earlier reported by Cronauer and Krikorian (1987) in banana and Reghunath (1989) in cardamom, were formed at higher concentrations of BA. Regeneration of calloids were observed in the same media and the maximum multiple shoots were produced in the treatment involving MS^a containing kinetin 5 ppm + NAA 1 ppm.

Somatic organogenesis was induced and shoots were formed when calloid tissues were subcultured onto an auxin-free medium containing BA 3.0 ppm + KIN 0.5 ppm. Cronauer and Krikorian (1987) observed adventitious shoot productions from calloid cultures from multiplying shoot cultures of triploid dessert clones like Philippine Lacatan and Grande Naine, cultured on MS media with added iron, sucrose, myoinositol and growth regulators. Although roots were induced by removing the kinetin, no shoot or plantlets were recorded.

In pineapple, Wakasa (1978) reported the differentiation of plants from protocorm like callus. Differentiation of meristemoids from hybrid embryo callus occurred on MS medium with 2.0 ppm NAA, 2.0 ppm IBA and 2.5 ppm BA (Rao et al., 1981). In the present study calloids produced maximum shoots on MS^a and MS^b medium containing KIN 5 ppm + NAA 1 ppm. Roots were produced in MS^b medium + NAA 2 ppm + AC 0.05 per cent and plantlets were successfully transplanted.

Deambrogio and Dale (1980) comparing the regeneration of shoots from callus and calloid reported that the failure of callus produced in regenerating shoots may be attributed to the progressive domination of polyploid cells in callus. Mitra et al. (1960) had earlier reported that only diploid cells of callus can regenerate shoots. According to them in calloids, there is a lesser proportion of polyploid cells and domination of diploid cells, which may favour the regenerating capacity of calloids.

5.5 Somatic organogenesis (callus mediated)

The most remarkable advantage of the method of producing shoots/plantlets through enhanced release of axillary buds is the genetic stability of the plantlets produced (Chand and Roy, 1980; Rao and Lee, 1986). But as far as the rate of plant multiplication and the induction of genetic variation are concerned, somatic organogenesis (callus mediated) and embryogenesis are reported to have greater potentialities than axillary bud release method. In the present study attempts were made to induce somatic organogenesis (callus mediated) and emrbyogenesis in Musa sp. (AAB) 'Nendran'. Various explants viz. shoot tip, inflorescence axis, flower base, embryonic leaves and scalp (in vitro) were tried for initiating callus. Bakry and Rossignol (1985) tried various explants of banana for analysing the callus formation and organogenesis and found that only floral or inflorescence tissues produced calli, but no organogenesis was reported to have occured Jarret et al. (1985) reported that spherical callus were developed on the surface and within leaf base explants of banana. Thin, meristematic layers excised from proliferating shoot tip cultures (scalp) of Musa AAB cv. Bluggoe produced callus (Banerjee et al., 1985).

Within any plant, tissues differ in their degree of determination and in their ability to undergo morphogenesis. Takayama and Misawa (1980) examined the ability of differing explants of Lilium auratum and L. speciosum to produce bulbs in vitro. Fifty per cent

of the peduncle explants, 75 per cent of the petal explants and 95 per cent of the bulb scale explants produced bulbs. Leaf explants and explants from stamens and anthers did not even survive the culture conditions. When the explant was taken from bulbs grown in culture, the success rate was 100 per cent. The conditioning effect, that is, an improved morphogenic response with explants from plants grown in culture has been noted in other species including several species of Gesueriaceae (Hughes, 1981).

Shoot tip was also taken as an explant in callus induction studies of banana (Jarret et al., 1985; Cronauer and Krikorian, 1987; Huang and Chi, 1988; Dhed et al., 1991).

When flower shoot tips of Dwarf Cavendish banana plants were cultured on amended MS medium, small amounts of nodular white callus were induced. The amendments comprised NaH_2PO_4, L-tyrosine, glucose and BA (Fitchet, 1986).

In the present study, of the various explants tried viz. shoot tip, inflorescence axis, flower base, embryonic leaves and scalp, embryonic leaves and scalp explants showed potentialities for rapid callus formation. Youngest and less differentiated tissues are found in plant meristems and culture of this plant tissue has been successful in a wide range of species (Hughes, 1981). Likewise, the absence of highly meristematic region in the explants other than embryonic leaves and scalp explants, may be the reason for the low response to callusing.

After selecting the explant, the basal medium for callusing was tried. Different researchers had tried different media for culture establishment. Callus was obtained from immature zygotic embryos of diploid species of Musa using a medium derived from MS (Escalant and Teisson, 1989). Proembryogenic calluses were initiated from basal leaf sheaths and rhizome tissue on modified Shenk and Hildebrandt medium with 30 µM dicamba (Novak et al., 1991). In the present study, it was observed that, of the media tried viz., MS^a, MS^b, LS and SH, MS^b was found to be more effective for culture establishment closely followed by MS^a.

In general, monocotyledonous species do not show a pronounced response to cytokinins and require high concentrations of potent auxins such as 2,4-D to achieve changes in the development of cultured tissues (Harms, 1982). Taking MS^b as basal medium, the best combination of auxins (2,4-D, 2,4,5-T) and cytokinin (BA) for different responsive explants were standardised. Dodds and Roberts (1985) reported that addition of a cytokinin along with auxins may be helpful in achieving better callusing. In the present study, 2,4-D was found to be the best for callus induction compared to 2,4,5-T. A combination of 2,4-D, 5 ppm and BA 1 ppm was observed as more effective for callus induction of shoot tip and embryonic leaves. For scalp explants, when the concentration of 2,4-D was increased (7 ppm) rate of callus induction was also increased. The use of a combination of 2,4-D and BA in callus induction by culturing of scalp has been reported by Dhed et al. (1991).

Response of the explants to callus formation was generally poor. Out of the total callus index assigned, none of the explants could record a CI value higher than 266. Although several investigators have attempted to establish callus and subsequently cell suspensions of banana this work has met with very limited success. Mohanram and Steward (1964), could get callus like growth from plugs of preclimateric fruit tissues. However the culture proved to be non-morphogenic. Rao et al. (1982) have reported the establishment of callus cultures from discs of the inflorescence axis. The callus could be induced to form roots, but no shoot or bud formation was observed. Bakry and Rossignol (1985) tried various explants of banana and analysis the callus formation and organogenesis. Only floral tissues produced calli, but no organogenesis occured It was noted that Reghunath (1989), eventhough treatments involving 2,4-D produced friable callus, they did not initiate shoot organogenesis. Occationally root organogenesis was observed in certain cultures. Srytanova and Mukhitdinova (1984) and Fitch and Moore (1984) also made similar observation in liquorice and sugarcane respectively. In the present study conducted to observe the effect of auxin and cytokinin on callus differentiation, rhizogenesis was observed from the callus of shoot tip explants, in the treatment involving combination of 2,4-D and BA, BA alone and basal MS media. In another experiment conducted with different levels of nitrate source for callus differentiation no organogenesis was recorded. Thus the study conducted for callus differentiation

resulted into the formation of roots and embryoid like structures and no shoot or buds were recovered.

The . failure of callus, produced in 2,4-D containing medium to regenerate shoots may be attributed to the progressive domination of polyploid cells in the callus (Deambrogio and Dale, 1980). Mitra et al. (1960) reported that only diploid cells of callus can regenerate shoots and as the percentage of polyploid cells increase, the regeneration capacity decreases.

5.6 Somatic embryogenesis

Somatic embryoids of triploid clones Saba and Pelipita were produced in liquid culture medium from cells derived from multiplying shoot tip cultures (Cronauer and Krikorian, 1983). Banerjee et al. (1985) reported callus induction from thin meristematic layers excised from proliferating shoot tip cultures of Musa (AAB) cv. Bluggoe when cultured on MS basal medium supplemented with 2,4-D or 2,4,5-T on prolonged incubation. The callus developed numerous globular white proembryogenic masses all over the surface upon transfering to MS liquid medium devoid of 2,4-D and 2,4,5-T. The proembryoids obtained from 2,4-D produced roots while those from 2,4,5-T regenerated bipolar embryoids. The same result was observed in the present study conducted for somatic embryogenesis but regeneration of embryogenic globules were not obtained.

5.7 Hardening and acclimatization

Hardening the *in vitro* raised plantlets so as to make them adapt to the outside environment is a critical process due to their anatomical and physiological peculiarities. On transplanting excessive water loss from the plants had been recorded which was attributed to the improper development of cuticle and slowness of stomatal response to water stress (Brainerd and Fuchigami, 1981; Fabbri *et al.*, 1984). The problem may be aggravated if the vascular connection between root and shoot is improper. A period of humidity acclimatization was considered necessary for the newly transferred plantlets to adapt to the outside environment, during which the plantlets undergo morphological and physiological adaption enabling them to develop typical terrestrial plant water control mechanism (Grout and Aston,1977; Sutter *et al.*, 1985). In the present study high relative humidity (90 to 100 per cent) was maintained during the initial period of planting out with the help of microscope and plastic covers with intermittent cold water sprays.

According to Bhaskar (1991), out of the various hardening treatments adopted, maximum percentage of plantlets survived when the plantlets after removal from the rooting media were kept in distilled water for 8 h and then for another 8 h in MS liquid medium of 1/10th strength under moistened microscope cover and after planting out into the potting media covering with moistened

plastic cover for four weeks. In the present study, the same above procedure as above was adopted for hardening the plantlets, except that before transferring them into potting media, root of the plantlets were dipped in 0.1 per cent Bavistin for 5 minutes. After planting the plantlets were covered with microscope cover. The hardening treatments resulted in better results.

The most suitable potting medium for maximum plant survival (90 per cent) was found to be sand. Sand might have helped the plant in giving a better grip for the roots besides providing ample aeration. Addition of inorganic nutrients to the potting mixture is essential for the normal growth of the potted plants (Brown and Sommer, 1982; Amerson et al., 1985). Application of 5-10 ml nutrient solution containing MS inorganic salts at half concentration at weekly intervals enhanced the survival and promoted normal growth of the plantlets. Rajmohan (1985) and Reghunath (1989) also reported the use of nutrient solution similar to the above for the successful growth of in vitro raised jack and cardamom plantlets respectively.

5.8 Screening for somaclonal variation

Repeated subculturing in vitro has been reported to increase the chance of genetic variation (Pierik, 1987). Evidence from cytological and molecular studies show that plant genome is relatively unstable and subject to various changes in developing somatic tissues. Larkin and Scowcroft (1981) reported numerical or structural changes

in chromosomes in association with *in vitro* regeneration of plants. Numerical changes have been observed in callus cultures of several crops (Evans and Sharp, 1983). Gene amplification has been shown to occur in plants grown in stressful environments (Cullis, 1975). The variability reflected either pre-existing cellular genetic difference or tissue culture induced variability (Evans *et al.*, 1984). D'Amato (1984) observed nuclear cytology of tissue cultures. When meristems or other plant explants are cultured on hormone containing media, a callus may be formed and genetic variation arises. Variation in somocromic structure and number in plant cells during *in vitro* tissue culture were investigated by Singh (1984). Though changes in ploidy level are not generally associated with repeated subculturing, the somatic chromosomes were counted in the root tips of plantlets from all the subcultures, to confirm the ploidy level. There was no variation in chromosome number and all the plantlets from all the subcultures were triploids ($2n = 33$).

The plantlets from different subcultures were planted out in sand, which was found to be the best medium and observations were recorded on growth parameters at fifteen days interval to find out the variation if any, induced by subculturing. Observations recorded after 60 days revealed that, plantlets from subcultures differed significantly in the rate of growth with respect to height as well as leaf area. Maximum growth rate with respect to height was recorded by the plantlets from the sixth subculture and

maximum growth rate with respect to leaf area was recorded by the plantlets from the fourth subculture. Ventura et al. (1988) observed somaclonal variation with respect to yield and vegetative characters in micropropagated bananas. Vuylsteke et al. (1988) found phenotypic variation among in vitro propagated plantain (Musa sp. cultivar AAB). Observations of the first crop and the successive ratoon indicated five different forms of phenotypic variation at a frequency of 6 per cent. Robinson and Nel (1989) found the highest mutation percentage in Dwarf Cavendish (3.33 per cent) and lowest in Grande Naine (0.63 per cent). Robinson (1989) observed vegetative morphology and phenology of tissue cultured banana plants. In the first cycle, tissue cultured plants produced 6-7 more leaves before flower emergence than did plants from suckers and they reached the flowering stage 15 and 24 days earlier. Variation in inflorescence types, in African plantains was reported by Vuylsteke and Swennen (1990) who noted that much of this somaclonal variation was enhanced in vitro. Quantitative aspects of somaclonal variation was reported by Israeli et al. (1991). Somaclonal variants of seven in vitro propagated banana cultivars were expressed in plant stature, abnormal leaves, pseudostem pigmentation, persistance of flowers and split fingers. Dwarfism was the most common variant indicated by mutation of intermediate height cultivars to a Dwarf stature.

The use of somaclonal variation in cultivar improvement has already advanced to the stage of field testing of promising variants in sugarcane, potatoes and pasture species. Some horticultural

cultivars of Pelargonium and Begonia have been derived from cell culture variants. Induction of somaclonal variation by utilization of *in vitro* techniques should substantially contribute to the broadening of the spectrum of genetic variation among *Musa* clones with obligate vegetative reproduction. Integration of methods of early screening for disease resistance (Stover, 1986; Epp, 1986; Hwang, 1986; Mourichon *et al.*, 1987) with subsequent rapid propagation of desirable plants leads to an alternative breeding scheme, which should be applied for banana improvement.

Summary

SUMMARY

The results of the studies on the induction of variability in Musa sp. var. 'Nendran' by in vitro methods are summarised in this chapter. In the study, the explants namely, shoot tip and eye bud were regenerated through enhanced release of axillary buds and, shoot tip, embryonic leaves, flower base, inflorescence axis and scalp were regenerated through a callus phase by somatic organogenesis/embryogenesis.

1. For shoot tip and eye bud explants, the sterilization treatment which resulted in least contamination was an initial dipping of explants in (0.1 per cent) emisan for 30 minutes followed by dipping in (0.1 per cent) norfloxacin for 30 minutes and finally rinsing in (0.1 per cent) mercuric chloride for 20 minutes.

2. The addition of activated charcoal into the media at the rate of 500 mg per litre reduced media and explant discolouration caused by polyphenol oxidation.

3. For the better and speedier establishment and growth of shoot tip and eye bud explants, MS (semi-solid) medium containing NAA 2 ppm and BA 5 ppm, and NAA 2 ppm and KIN 10 ppm, respectively, were found to be the best. Compared to eye bud,

shoot tip explants took lesser time for culture establishment. In shoot tip culture, on an average each explant released 8.66 axillary shoots when MS^b contained NAA 2 ppm + BA 10 ppm. In the case of eye bud explant, on an average each explant released 5 shoots, when MS medium contained NAA 2 ppm and BA 5 ppm.

4. Shoot multiplication rate increased on an average by 5.9 due to repeated subculturing at two weeks intervals continuously for ten serial subcultures.

5. Hard greenish globular callus like structures viz. calloids was produced in the treatment involving MS medium containing BA 10 ppm. Regeneration of calloid was found in the same medium. On an average each calloid culture released four axillary shoots in MS^a and MS^b media containing KIN 5 ppm + NAA 1 ppm.

6. For <u>in vitro</u> rooting, maximum number (5.67) of roots per shoot was recorded by MS^a medium containing NAA 5 ppm and AC 0.05 per cent.

7. For somatic organogenesis (callus mediated) scalp explant registered maximum response within a minimum time compared to shoot tip, inflorescence axis, flower base and embryonic leaf explants. For shoot tip and scalp explants, MS^b medium containing 5 ppm 2,4-D and 1 ppm BA recorded maximum callus initiation. In the case of embryonic leaves, treatment involving the

same medium containing 2,4-D 7 ppm and BA 1 ppm registered maximum response to callusing.

8. Maximum callus index (266) was recorded by scalp explants followed by embryonic leaves (216.6).

9. Rhizogenesis was observed from the callus of shoot tip explants. In MS^a media, rhizogenesis was recorded in the treatment involving basal media of liquid and semi-solid consistency. In MS^b media rhizogenesis was recorded by the treatments involving BA 5 ppm (semi-solid) and BA 1 ppm + 2,4-D 5 ppm (liquid and semi-solid consistency). Emrbyoids were produced by scalp explants, which was observed in the treatment involving MS + 2,4-D 2 ppm of liquid consistency.

10. There was no significant effect of different levels of nitrate source on callus differentiation.

11. After removal from the rooting media, plantlets were kept in distilled water for 8 h and then for another 8 h in liquid MS medium (containing 1/10th strength of inorganic salts, with no sucrose) under microscope cover.

12. After the hardening treatments, growth parameters of plantlets derived from different subcultures were recorded. With respect to height, maximum result was (12.50 cm) recorded by 10th sub-culture, number of leaves produced was 2/3 in all the subcultures, maximum length (6 cm) of the leaf was recorded by 9th and

10th subcultures, number of roots per shoot was maximum (7) at 5th subculture and length of the roots were maximum (13 cm) at 6th and 7th subcultures.

13. Somatic chromosome counts made at the root tips of plantlets from 10 subcultures, revealed that all the plants were triploids (2n = 33).

14. The most suitable potting medium giving maximum plant survival percentage (90) was found to be sand.

15. The plantlets from different subcultures were planted out in sand, which was found to be the best medium and observations were recorded on growth parameters at fifteen days interval to find out the variation if any, induced by series of subculturing. The plants from subcultures differed significantly with respect to growth rate of plant height and leaf area. Maximum growth rate (1.03 cm) with respect to height was recorded by 6th subculture and maximum growth rate (19.2 cm^2) with respect to leaf area was recorded by 4th subculture.

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INDUCTION OF GENETIC VARIABILITY IN *Musa Sp.* Sp. Var. NENDRAN BY *in vitro* METHODS

By

MINI BALACHANDRAN

ABSTRACT OF A THESIS

Submitted in partial fulfilment of the
requirement for the degree

Master of Science in Horticulture

Faculty of Agriculture
Kerala Agricultural University

1993

ABSTRACT

Investigations were carried out at the Department of Pomology and Floriculture and Plant Tissue Culture Laboratory of the All India Co-ordinated Floriculture Improvement Project, College of Horticulture, Vellanikkara, Thrissur, during 1991-93 on the induction of variability in the banana variety Nendran (Musa AAB 'Nendran') by in vitro methods. Explants utilized for the study were shoot tip and eye bud for direct organogenesis through enhanced release of axillary buds and shoot tip, flower base, inflorescence axis, embryonic leaves and scalp for somatic organogenesis/embryogenesis. For culture establishment, axillary shoot initiation and in vitro rooting, different growth regulators, like NAA, 2,4-D and 2,4,5-T (auxins) and BA and kinetin (cytokinins) were made use of. The plantlets produced in vitro were subjected to hardening treatments to ensure a better establishment of planted out plants and their growth parameters were studied.

For shoot tip and eye bud explants, a combination of treatments involving, an initial dipping of explants in emisan (0.1 per cent) for 30 minutes followed by dipping in norfloxacin (0.1 per cent) for 30 minutes and finally rinsing the explants in mercuric chloride (0.1 per cent) for 20 minutes was found to be best, but for flower base and inflorescence axis explants, emisan (0.1 per cent) treatment for 20 minutes and for embryonic leaves, dipping in alcohol for one second were the best. Better and speedier

establishment and growth of shoot tip and eye bud explants were observed on MS (semi-solid) medium containing NAA 2 ppm + BA 5 ppm.

Addition of activated charcoal (500 mg per litre) to the medium, reduced media and explant discolouration due to polyphenol oxidation. When the performance of the shoot tip and eye bud explants was compared, eye bud explants took more time for culture establishment and growth.

In shoot tip culture, on an average, each explant released 8.66 axillary shoots in the treatment involving MS^{b*} + NAA 2 ppm + BA 10 ppm. In the case eye bud, on an average, each explant released five axillary shoots.

Continuous subculturing was carried out at two week interval to assess the variation induced to cultured plants due to repeated subculturing. It was found that, the number of shoots produced per culture was not constant in all the subcultures. Still, the axillary shoots produced per explant per culture vessel increased at the mean rate of 5.90.

BA alone at higher concentration (10 ppm) resulted in calloid (globular semi-hard, light green callus like structure) formation and subsequent regeneration

MS^{b*} : MS medium containing half concentration of inorganic salts and full concentration of organic growth factors

For in vitro rooting, MS^{b} medium containing NAA 10 ppm and AC 0.05 per cent was found to be effective for early root initiation and the maximum number of roots per shoot was produced at the treatment involving MS^{a*} + NAA 5 ppm + AC 0.05 per cent.

Of the various explants, viz., shoot tip, inflorescence axis, flower base, embryonic leaves and scalp (in vitro) tried for initiating callus, scalp and embryonic leaves recorded maximum response. Among the media tried for callus initiation, MS^{b} media at liquid consistency was found to be more effective. Maximum callus index (266) was recorded for the treatment combination involving 2,4-D 7 ppm and BA 1 ppm. For callus differentiation the treatments involving 2,4-D and BA, BA alone and basal MS media resulted in rhizogenesis, and treatments involving 2,4-D alone produced embryoid like structures from scalp callus. No shoot organogenesis was observed. Also treatments were conducted with changed levels of nitrate source in the media, but they did not give any favourable results. Embryoid like bipolar structures were recovered from scalp callus when they were transferred to media devoid of growth regulators.

To study the variation, if any, induced due to serial subculturing, the shoots obtained from each subculture cycle (through enhanced release of axillary buds) were isolated and their

MS^{a*} : MS medium containing full concentration of inorganic salts and organic growth factors

identity maintained. The shoots thus separated were rooted and planted out after subjecting them to a process of hardening. Somatic chromosome counts were made at the root tips of plantlets from 10 subcultures to confirm the ploidy. All the plants were triploids (2n = 33). The plantlets from different subcultures were planted out in sand, which was found to be the best medium. Observations made on growth parameters, at fifteen days interval, revealed that the plants from subcultures differed significantly with respect to the rate of growth in height and leaf area.

Appendix

Abstract of analysis of variance for the effect of different treatments

Treatment/character	Mean square		Level of significance (%)
	Treatment	Error	
1	2	3	4
A. Effect of media supplements on the discolouration of media and explant			
i) Death of culture due to contamination	270.48	31.38	5
ii) Death of culture due to discolouration	1103.15	43.61	5
iii) Percentage of culture survival	813.02	32.27	5
B. Effect of combination of auxin and cytokinin on culture establishment			
a. NAA + BA			
I. Days taken for culture establishment			
i) Shoot tip	51.70	2.66	5
ii) Eye bud	8.44	2.50	5
II. Days taken for culture elongation			
i) Shoot tip	8.11	1.08	5
ii) Eye bud	7.37	1.83	5
b. NAA + KIN			
I. Days taken for culture establishment			
i) Shoot tip	0.81	1.33	5
ii) Eye bud	3.00	1.41	5
II. Days taken for culture elongation			
i) Shoot tip	1.38	2.12	5
ii) Eye bud	3.15	1.41	5

Contd.

Appendix Continued

1	2	3	4
C. Effect of auxin (NAA) and cytokinin (BA) on culture proliferation			
I. Percentage of culture developing shoots			
i) Shoot tip	58.97	52.38	NS
ii) Eye bud	156.04	72.61	5
II. Shoots/culture			
i) Shoot tip	14.74	1.92	5
ii) Eye bud	3.24	0.88	5
D. Effect of repeated subculturing			
a. Shoots/culture	246.45	176.36	NS
E. Rooting			
a. Days taken for root initiation	5.72	1.05	5
b. Number of roots/shoot	5.05	1.66	NS
F. Effect of repeated subculturing on growth rate			
a. Height	0.33	0.04	5
b. Girth	0.06	0.10	NS
G. Somatic organogenesis			
a. Culture establishment			
I. Percentage of culture survival	141.29	14.37	5
II. Percentage of culture initiating callus	708.89	70.02	5
III. Days taken for callus initiation	14.48	2.51	5