In vitro Mass Propagation and *Agrobacterium*-mediated Genetic Transformation of Banana Plants Native to Mizoram

Thesis
submitted to Mizoram University
in partial fulfilment for the award
of
Doctor of Philosophy in Biotechnology

By

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Registration no. & date: MZU/Ph.D/253 of 28.11.2008

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Certificate

This is to certify that the present thesis entitled "***In vitro* mass propagation and *Agrobacterium*-mediated genetic transformation of banana plants native to Mizoram**" submitted by **Miss Lalremsiami Hrahsel**, Ph.D Registration No. MZU/Ph.D/253 of 28.11.2008 in partial fulfillment for the award of the Degree of Doctor of Philosophy in Biotechnology of Mizoram University has been carried out during 2008-2013 under my supervision. The thesis embodies original research work and has not been submitted for any degree elsewhere.

The candidate has fulfilled all the requirements under Ph. D. ordinance of the Mizoram University.

(Dr. Thangjam Robert Singh)
Supervisor

Declaration of the Candidate

I, Lalremsiami Hrahsel, a Ph.D scholar in Biotechnology Department, Mizoram University, Aizawl, do hereby solemnly declare that the subject matter of this thesis is the record of the work done by me. I have duly worked on my Ph.D thesis under the supervision of Dr. Thangjam Robert Singh, Assistant Professor, Department of Biotechnology, Mizoram University. This is being submitted to Mizoram University for the award of the degree of Doctor of Philosophy in Biotechnology and that I have not submitted this work to any other University or Institute for any other degree.

I also declare that the present investigations were related to the bonafide research works undertaken for the thesis entitled "*In vitro* Mass Propagation and *Agrobacterium*-mediated Genetic Transformation of Banana Plants Native to Mizoram".

(DR.THANGJAM ROBERT SINGH) (LALREMSIAMI HRAHSEL)

Supervisor *Candidate*

God for His glory,

To my beloved parents and brothers,

And to the people of Mizoram

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Date: *(LALREMSIAMI HRAHSEL)*

ABBREVIATIONS

2-ip	2-isopentyl adenine
2,4- D	2,4-Dichlorophenoxyacetic acid
AdS	Adenine Sulphate
ANOVA	Analysis of variance
B5	Gamborg's medium (1968)
BAP	6-benzylaminopurine
CAMBIA	Centre for Application of Molecular Biology to International Agriculture
CaMV	Cauliflower Mosaic Virus
CLB	Cauliflower-like bodies
CTAB	Cetyltrimethyl ammonium bromide
DMRT	Duncan's multiple-range test
DNA	Deoxyribonucleic acid
dNTP	Deoxy nucleotide triphosphate
ECS	Embryogenic cell suspensions
EDTA	Ethylenediaminetetraacetic acid
GA	Giberrelic acid
gus	β-1-4 glucuronidase
HCl	Hydrochloric acid
$HgCl_2$	Mercuric chloride
hpt	hygromycin phosphotransferase
IBA	Indole-3- butyric acid
IAA	Indole-3-acetic acid

ISSR	Inter Simple Sequence Repeat
Kin	Kinetin
LAF	Laminar air-flow chamber
LB	Luria-Bertani
MS	Murashige and Skoog’s medium (1962)
NAA	α-Naphthalene acetic acid
NaOCl	Sodium hypochlorite
NaOH	Sodium hydroxide
nptII	neomycin phosphotransferase II
OD	Optical density
PGR	Plant growth regulator
PCR	Polymerase chain reaction
RAPD	Randomly Amplified Polymorphic DNA
RNA	Ribonucleic acid
SAAT	Sonication-assisted *Agrobacterium*-mediated transformation
SDS	Sodium Dodecyl Sulphate
T-DNA	Tranferred-DNA
TDZ	Thidiazuron
Ti-plasmid	Tumer inducing plasmid
UBC	University of British Colombia
UV	Ultra Violet
vir	virulence gene
YMA	Yeast Mannitol Agar
Zea	Zeatin

UNITS

µg	microgram
µl	microliter
µm	micromolar
^{0} C	degree celsius
bp	basepair
cm	centimeter
g/L	gram per liter
kb	kilo base, kilo basepair
kDa	kilodalton
kHz	kilohertz
kg	kilogram
mg	milligram
mg/L	milligram per liter
min	minute
ml	milliliter
mM	millimolar
mm	millimeter
mm Hg	millimeters of mercury
pH	negative log of H+ ion
psi	pounds per square inch
rpm	Revolution per minute
Rs	rupees
s	second
v/v	volume/volume (concentration)
w/v	weight/volume (Concentration)
V	Volt

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Chapter 1

Introduction

CHAPTER 1

INTRODUCTION

1.1. General Introduction

Bananas (*Musa* spp.), including dessert and cooking types, are giant perennial monocotyledonous herbs under the order Zingiberales belonging to the family Musaceae (Hont *et al*., 2012). According to Li *et al*. (2010b) Musaceae contains only two genera *Musa* L. (Linnaeus, 1753) and *Ensete* Horan. (Horaninow, 1862) where *Musa* differs from *Ensete* in producing suckers and small seeds (Samson, 1992). Banana is distributed in more than 120 countries covering almost 10 million hectares, with an annual production of 95 million tonnes. It is the fourth most important food crop after rice, wheat and maize (Uma and Sathiamoorthy, 2002). Banana cultivars are mainly derived from *Musa acuminata* Colla (A genome) and *Musa balbisiana* Colla (B genome) which are sometimes diploid but generally triploid (D' Hont *et al*., 2000). The *Musa* domestication process started around 7,000 years ago in Southeast Asia. Through hybridizations between diverse species and subspecies and also fostered by human migrations, banana got dispersed across the tropics and sub-tropics by means of vegetative propagation. The selection of diploid and triploid seedless parthenocarpic hybrids thereafter helped in the process of its wide distribution (Perrier *et al*., 2011). The modern day edible bananas are a mix of wild and cultivated species and hybrids associated with *M. acuminata* and *M. balbisiana* while *M. acuminata* is the most widespread of the species in the section *Musa* (Daniells *et al*., 2001). Half of the current banana production relies on somaclones derived from the triploid Cavendish sub group (Lescot, 2011).

1.2 Status of banana

Southeast Asia is considered as the region where banana originated from and many of its wild relatives are native (Uma *et al.*, 2001). In India more than 970 indigenous bananas from all over the country were collected and maintained in the National Research Centre on Banana (NRCB), Trichy, Tamil Nadu. Out of these, 109 were collected from northeast states of the country, namely Assam, Arunachal Pradesh, Meghalaya, Tripura, Mizoram and Manipur. The northeastern region of India is considered to be the source of richest natural banana diversity (Uma and Sathiamoorthy, 2002). This region is also regarded as the reservoir for the large genepool of banana genetic resources and is the meeting point of *M. balbisiana* of the Indian subcontinent and *M. acuminata* of Southeast Asia (Molina and Kudamage, 2002). Altogether 39 different accessions of banana have been collected and characterized from the region (Uma *et al.*, 2001). From the state of Mizoram, 14 different accessions have been collected and characterized (Lalrinfela and Thangjam, 2012). With the loss of crop genetic resources at an alarming rate, the future of global food crops depend on the sustainability of the genetic pool at their centre of diversity.

1.3 Economic importance of banana

Bananas are multipurpose plants as most of their parts can be used in various ways depending on the species. Fruits can be eaten either ripe as dessert or cooked (Smith *et al.*, 2005). They are nutritionally rich in carbohydrates, vitamins A, B, and C, and potassium (Aurore *et al.*, 2009). The unripe fruit can also be brewed to prepare beer and wine or processed into sauce, flour, chips, crisps, smoked products and confectionaries. The unripe fruits are the good sources of amylase and starch (Van

den Houwe *et al*., 2000). Male floral buds can be eaten as vegetables whereas pseudostems are the sources of fiber for the manufacture of rope, paper and textiles. Banana leaves are used for thatching of roofs, in the production of fabric and cordage and as animal forage (Smith *et al*., 2005). Species such as *M. ornata* and *M. veluntina* are popular ornamental plants (Heslop-Harrison and Schwarzacher, 2007).

Global banana cultivation is focused mainly on parthenocarpic cultivars for the production of edible fleshy seedless fruits. Approximately 47% of the global banana production has been dominated by cultivars of the Cavendish type because of their high yields and short periods to maturity (Robinson, 1996; Kulkarni *et al*., 2007). The world banana-fruit production in 2007 was estimated to be 86 million tonnes, harvested from an area of about 5 million hectares. Banana was ranked second after citrus on the basis of the world fruit-crop production in 2007 (FAOSTAT, 2009). The production of banana is primarily for local consumption as a dietary supplement or staple food in the producing countries. An estimated 80% (68 million tonnes) of the world banana-fruit production in 2007 was consumed and traded locally in the producing countries. Consequently, fruit production is important to both food and income securities of the producing countries.

In 2007, the world export of banana, consisting mainly of Cavendish-type dessert banana, was estimated to be 18 million tonnes (20% of world production), amounting to US$ 7.2 billion in economic terms (FAOSTAT, 2009). The most important attributes that make the Cavendish subgroup the main bananas for export are related to their reliability during transport and their shelf life, rather than taste. In economic value, banana fruit ranked fifth in the world trade for agricultural crops (Aurore *et al*., 2009). Though India was the leading producer of banana in 2007 (Sipen *et al*., 2011),

the leading banana exporters were Colombia, Costa Rica, Ecuador, Guatemala, and the Philippines. This clearly indicates the high domestic consumption rate of banana in India.

1.4 Origin of the problem

Banana production all over the world has been seriously affected by several diseases and pests (Stover and Simmonds, 1987; Jones, 1999). Most of the problems were associated with plantation of poor quality clones in the field (Novak, 1992). Various major fungal diseases such as Sigatoka disease (SD) due to *Mycosphaerella musicola* and black leaf streak disease (BLSD) caused by *M. fijiensis* result in production losses in large plantation areas and necessitate costly pest control measures to be adopted. In certain production zones, *Fusarium* wilt due to *Fusarium oxysporum f.* sp. *cubense* prevents the cultivation of susceptible varieties like the Gros Michel types. Nematodes such *Radopholus similis* and several representatives of the genus *Pratylenchus* also lead to various problems in banana production along with the black weevil of banana (*Cosmopolites sordidus)*. Several viral diseases such as BBTV (banana bunchy top virus), CMV (cucumber mosaic virus), BSV (banana streak virus) and BBMV (banana bract mosaic virus) are spreading causing large scale damage of crops. In Northeast India banana diseases such as BBTV, Black Sigatoka and Yellow Sigatoka (*M. musicola*) are prevalent (Uma *et al*., 2001)

The development of new banana varieties through conventional breeding programs remains difficult because of the sterility and polyploidy of most edible cultivars. The conventional means of propagation is through suckers. However, the production of suckers varies in different genotypes ranging from 5-10 per plant per year. Crop productivity and maturity is also dependent on the size and age of suckers.Besides,

suckers usually carry soil nematodes, disease causing organisms such as bunchy top virus, leaf spot etc., thereby affecting the crop production considerably. In this regard, biotechnological approaches such as cell and tissue culture, protoplast fusion and gene transfer can serve as useful tools (Novak *et al*., 1993). Plant tissue culture and molecular genetic techniques have shown great potential to overcome some of the factors limiting traditional approaches to banana and plantain improvement. Such procedures largely depend on successful regeneration of plants from cultured banana cells and/or tissues. Therefore the integration of biotechnology into banana and plantain breeding programs require access to reliable cell and tissue culture protocols.

1.5 Biotechnological approach for banana improvement

As a result of the various shortcomings associated with banana propagation through vegetative means the application of various biotechnological approaches has become an integral part of the banana industry (Vuylsteke *et al*., 1998). In particular, the use of plant tissue culture *via* clonal propagation of superior cultivars has been an immense benefit to commercial banana farmers globally (Jain, 2004). *In vitro* propagation provides excellent advantages over traditional propagation including a high multiplication rate, physiological uniformity, availability of disease-free material all the year round, rapid dissemination of new plant materials throughout the world, uniformity of shoots, short harvest interval in comparison with conventional plants, and faster growth in the early growing stages compared to conventional materials (Vuylsteke, 1989; Daniells and Smith, 1991; Arias, 1992). Thus, tissue culture techniques can offer faster and reliable means of producing large number of genetically uniform clonal planting material within a short period of time. Tissue culture techniques have been widely used for the multiplication and conservation of

plant germplasm. However, the high multiplication rates which are usually achieved using *in vitro* culture techniques, lead to regular production of large number of plant materials. This creates the problems for the management of large *in vitro* collection. In addition, risk of losing material through contamination or human error is present during subculture. Thus, *in vitro* conservation is necessary to minimize the previously mentioned risks, in particular, to preserve the genetic integrity of the plant material. *In vitro* conservation of culture has been applied with varying degrees of success to wide range of species and culture system, and successful slow growth system were developed for different species. Production of synthetic seeds by encapsulating somatic embryos and vegetative propagules is rapidly becoming an applied technique with potential for mass propagation of elite plant species. In vegetatively propagated plants, synthetic seeds would allow direct planting of clonal varieties and may provide a means for maintenance of elite germplasm. Advantage of using artificial seeds include ease of handling, transportation and potential storage, higher scale- up potential and low cost production, and subsequent propagation (Rao *et al*.,1998). Synthetic seed thus may provide the only technology realistically amenable to the extensive scale-up required for the commercial production of elite clones (Bornmann, 1993). *In vitro* production of plants involves the application of plant growth regulator, such as auxin, for initiation. Nevertheless, these auxins are known to be associated with genetic instability in plants, a phenomenon called somaclonal variation (Karp, 1989; Cullis, 1992). Mostly somaclonal variation also occurs as responses to the stress imposed on the plant in culture conditions and are manifested in the form of DNA methylations, chromosome rearrangements and point mutations (Phillips *et al*., 1994). Although somaclonal variations may be used as a source for

variation to get superior clones, it could be a serious problem in plant tissue culture industry where the aim is to develop identical propagules of a desired variety resulting in the production of undesirable traits or plant off-types (Karp, 1993; Cassells *et al*., 1999). Scaling up of any micropropagation protocol is severly hindered due to incidence of somaclonal variations, so a stringent quality check in terms of genetic similarity of progeny becomes mandatory. Any system, which can significantly reduce or eliminate variation generated during tissue culture, can be of much practical utility. Traditionally, morphological description, physiological supervision, karyological analysis, biochemical estimations and field assessment were used to detect any types of genetic variations, but presently molecular markers have complemented over traditional methods to detect and monitor the genetic fidelity of tissue culture derived plantlets and variety identification. This is apparent in studies conducted to screen somaclonal variations produced in tissue cultured derived plants such as in turmeric (Salvi *et al*., 2001), Lillium (Varshney *et al*., 2001), Strawberry (Gaafer and Saker, 2006) and *Swertia chirayita* (Jhosi and Dhawan, 2007). Kaeppler and Phillips (1993) and Kaeppler *et al*. (1998) suggested that tissue culture represents a unique form of stress. Vuylsteke *et al*. (1991) had suggested that in genus *Musa*, the extent of somaclonal instability was a result of the interaction between the genotype and the tissue culture conditions. Randomly amplified polymorphic DNA (RAPD) (Williams *et al*., 1990) and inter simple sequence repeats (ISSR) (Zietjiewicz *et al*., 1994) markers have proved to be efficient in detecting genetic variations.

Development of new banana varieties through conventional breeding programs remains difficult because of sterility and polyploidy of most edible cultivars. Genetic transformation offers a viable means for introduction of agronomically important

genes into these cultivars. Therefore, an efficient transformation protocol is crucial for banana improvement. Genetic transformation, involving the introduction and stable integration of genes into the nuclear or plastid genomes with subsequent gene expression in transgenic or transplastomic plants, offers an additional approach for the genetic improvement of banana, particularly for those cultivars that are not amenable to sexual hybridization, e.g., those from the Cavendish subgroup (Pillay and Tripathi, 2007). Both particle bombardment (Becker *et al*., 2000) and *Agrobacterium*-mediated gene-transfer techniques have been used to introduce foreign genes into banana (Ganapathi *et al*., 2001a; Khanna *et al*., 2004; Acereto-Escoffie *et al*., 2005). With respect to genotype specificity, Arinaitwe *et al*. (2004) compared the two transformation systems, *Agrobacterium*—and particle bombardment-mediated transformation for the generation of transgenic plants from embryogenic cell suspensions of four different banana cultivars ('Grand Naine' AAA, 'Three Hand Planty' AAB, 'Obino I'Ewai' AAB, and 'Orishele' AAB), and found that transient and stable gene expression were significantly higher in AAA with *Agrobacterium* method and is feasible for most banana cultivars. The genetic transformation technique has the potential to make a significant contribution to banana improvement. An important aspect of banana transformation is the fact that there is little chance of unintentional gene flow from transformed plants because of their sterility or extremely low fertility, making them particularly environmentally safe (Smith *et al*., 2005; Sunil *et al*., 2005; Pillay and Tripathi, 2007). In general, transformation frequencies are reported to be cultivar dependent. Thus there is a need to develop optimal transformation protocols for any particular genotype or cultivar of banana.

1.6 Objectives

The state of Mizoram (21˚58' to 24˚35' N - 92˚15' to 93˚29' E) is located in the northeastern region of India with an area of 21081 sq. Km. The geographical area of the state enjoys the tropical, sub-tropical and temperate agro-climatic conditions. Wild and edible banana varieties are abundantly distributed throughout Mizoram. Taking the mentioned biotechnological strategies into consideration for banana improvement of local varieties of Mizoram, and understanding the level of their genetic diversity will provide useful information for the status, breeding purposes and in producing uniform superior planting for large scale plantation. In spite of the availability of many reports on *in vitro* propagation in bananas, in which the protocols are complicated, the standardization of specific protocols for a specific cultivar is essential. Therefore, the present research work was conceptualized to address the scientific gaps for the identification of local banana cultivars of Mizoram and standardize the mass propagation of quality planting materials and genetic transformation using biotechnological approaches.

Objectives:

1. To identify and evaluate the commercially important edible banana cultivars native to Mizoram
2. To standardize the *in vitro* regeneration procedure for rapid multiplication of selected banana cultivars using different explants
3. To standardize the protocol for developing encapsulated seeds for long term *in vitro* storage
4. To standardize an effective *Agrobacterium*-mediated transformation system

Chapter 2

Review of Literature

CHAPTER 2

REVIEW OF LITERATURE

2.1 *In vitro* regeneration and synthetic seed production in banana

The first report of banana tissue culture was from Taiwan when shoot-tips were cultured to regenerate into *in vitro* adventitious buds (Ma and Shii, 1972; 1974) followed by Berg and Bustamante (1974) who used meristem culture combined with thermotherapy for the production of virus free banana plants. Since then numerous protocols have been achieved in banana tissue culture for enabling increase banana production and improvement.

There have been several reports on the use of different explants for the initiation of banana culture. *In vitro* propagation of banana employing the shoot tip culture or sucker has been practiced in many of the commercial cultivars (Kulkarni *et al.* 2004; 2006). Somatic embryogenesis has been successful with limited numbers of banana cultivars such as cv. Bluggoe (ABB) (Novak *et al.*, 1989; Dheda *et al.*, 1991; Panis *et al.*, 1993); cv. Grand Naine (AAA) (Novak *et al.*, 1989; Escalant *et al.*, 1994; Côte *et al.*, 1996; Becker *et al.*, 2000); cv. Rasthali (AAB) (Escalant *et al.*, 1994; Ganapathi *et al.*, 2001b) and cv. Mas (AA) (Jalil *et al.*, 2003). The banana explants that have been used in somatic embryogenesis studies are proliferating meristems (Cronauer-Mitra and Krikorian, 1983); zygotic embryos (Cronauer-Mitra and Krikorian, 1988; Escalant and Teisson, 1989; Marroqin *et al.*, 1993); rhizome leaf sheaths (Novak *et al.*, 1989) and female flowers (Grapin *et al.*, 2000). There are also many reports on the use of immature male flowers of banana with the formation of embryogenic cell suspensions (Escalant *et al.*, 1994; Côte *et al.*, 1996; Grapin *et al.*, 1996; Navarro *et*

al., 1997; Sagi *et al*., 1998; Becker *et al*., 2000; Jalil *et al*., 2003; Ghosh *et al*., 2009; Morais-Lino *et al*., 2008) while few reports on the use of immature male flowers of banana for direct regeneration are available (Table 2.1) Sidha *et al*. 2007 reported that among the explants that have been used for banana *in vitro* regeneration, immature male flowers and scalps are the most responsive starting material, for initiating regenerable and embryogenic cell suspension cultures. Considering the need to optimize culture protocols for diverse Indian banana cultivars, there have been efforts to establish regenerable cultures (Ganapathi *et al*., 2002; Suprasanna *et al*., 2002; Kulkarni *et al*., 2006). However, regeneration *via* callus phase has been proved to be less desirable for *in vitro* plant recovery due to high frequencies of genetic distortions (Sahijram *et al*., 2003; Martin *et al*., 2006; Ray *et al*., 2006).

Contamination and browning of the explant tissues is considered as constraints in the technique of *in vitro* culture for many motocotyledonous and woody species. For *in vitro* culture initiations explants are normally collected from field grown plants, hence the plant material is liable to be contaminated by microorganisms which must be disinfected before explants are transferred to *in vitro* conditions. Variations in sterilization procedures have been proposed by many researchers. Sodium hypochlorite or Clorox (commercial solution of 5.25% sodium hypochlorite) is the most common disinfectant for surface sterilization of banana explants (Krikorian *et al*., 1993; Abeyaratne WM and Lathiff MA, 2002; Clayton *et al*., 2003). Some other investigators have replaced sodium hypochlorite with low concentration of mercuric chloride (Sebastian and Matthew, 2004; Titov *et al*., 2006; Resmi and Nair, 2007). Sometimes explants are treated with fungicides and antibiotics to minimize the contamination in *in vitro* cultures (Van den Houwe, 1998; Nandwani *et al*., 2000).

Treatment of banana explants with 70% alcohol alone (Sidha *et al*., 2006; Pérez-Hernández JB and Rosell-García, 2008; Darvari *et al*., 2010) or along with sodium hypochlorite/mercuric chloride has also been used by many researchers (Madhulatha *et al*., 2006; Kalimuthu *et al*., 2007; Mahadev *et al*., 2011).

Also, many monocotyledonous and woody species are faced with a deleterious effect of oxidized phenols (Khatri *et al*., 1997, Zweldu and Ludders, 1998) accompanied by phenolic exudation in the culture medium. This process is initiated by browning of the surface of plant tissues due to the oxidation of phenolic compounds resulting in the formation of quinones which are highly reactive and toxic to plant tissue (Taji and Williams, 1996). The browning of the explant tissues at the beginning is the main difficulty as contamination can be controlled with proper handling and surface sterilisation techniques. Banana tissues are known to contain large amount of latex and phenolic compounds (Wu and Su 1990). Titov *et al*. 2006 found that inflorescence tissues of cv. Kanthali was high in phenolic compounds. Understanding the processes contributing to the oxidation of phenols and how these can be minimized when initiating explants is critical for successful *in vitro* culture and subsequent regeneration. The effectiveness of ascorbic acid in resolving the lethal browning problem of cv. Formosana plantlets was studied (Ko *et al*., 2009), where it was observed that ascorbic acid may have been absorbed by the plantlets, translocated to leaves, and prevented the oxidation of phenolic compounds on the target side. Ascorbic acid not only can prevent the occurrence of lethal browning in subsequently produced plantlets, it can also stop the progress of browning in affected plantlets.

For the standardization of the *in vitro* regeneration of banana, different media were analyzed. Some of the reports were on the initiation and multiplication of cultures in

the same media while others use low concentrations of hormones for culture initiation. The most common salt mixtures used for culture initiation of banana is MS media (Murashige and Skoog, 1962) with some modifications as reported by many authors (Hwang *et al*., 1984; Drew *et al*., 1989; Cronauer and Krikorian, 1984; Hamill *et al*., 1993; Thomas *et al*., 1995; Nandwani *et al*., 2000; Zaffari *et al*., 2000; Assani *et al*., 2003; Molla *et al*., 2004; Roels *et al*., 2005).

Plant growth regulators (PGRs) are essential media components for the manipulation of growth and development of explants *in vitro*. Their concentration and ratio in the medium often determines the pattern of development *in vitro*. Cytokinins and auxins are used as growth regulators for *Musa* tissue culture. The most widely used PGRs are 6-benzylaminopurine (BAP), α-Naphthalene acetic acid (NAA), Kinetin (kin) and thidiazuron (TDZ) (Harirah and Khalid, 2006; Darvari *et al*., 2010; Mahadev *et al*., 2011).

In a number of plant species, encapsulation and plant regeneration from synthetic seeds has been reported (Rao and Suprasanna, 1999). Plants were regenerated from synthetic seeds in carrot, celery, alfalfa, sandalwood, eggplant, asparagus, rice, barley and papaya (Rao *et al*., 1998). Sodium alginate has been the choice of gelling agent and calcium chloride the complexing agent for encapsulation experiments. Banana and plantain are the most important fruit crops in the world and viable seeds are not produced in edible triploid bananas. The number of suckers produced in a year is limited, and hence tissue culture propagation has become a practice all over the world.

Various *in vitro* regeneration protocols including encapsulation of the somatic embryos has been reported in banana using different media and hormone combinations (Marroquin *et al*., 1993; Ganapathi *et al*., 1992, 2001b; Navarro *et al*.,

1997). Ganapathi *et al*. (2001b) encapsulated somatic embryos of cv. Rasthali in 5% sodium alginate to produce synthetic seeds and seeds cultured on full strength MS medium showed maximum conversion frequency 66%.

Table 2.1 Available reports on direct *in vitro* regeneration of different banana cultivars using immature male flower as explants.

Cultivar	Surface sterilization	Media + Plant growth regulators + supplements utilized	Remarks	Authors
Chandrabale (Red banana), Rasthali, Robusta	-	MS basal medium; ADS, BA, IAA, IBA, NAA.	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*	Swamy and Sahijram, 1989
Maricongo, Superplatano	0.25% NaOCl with few drops of Tween 20 – 5 mins	MS liquid media; BAP	CLB (Cauliflower-like bodies are formed)	Krikorian *et al.*, 1993
Poovan (AAB), Nendran (AAB)	0.1 % mercuric chloride solution for 5 mins	MS media; BAP, AdS, GA_3; Effect of NaH_2P0_4 tested.	4.16% of the inflorescence tip derived plants transferred to the field demonstrated dwarfism in the field.	Sebastian and Matthew, 2004
Berangan (AAA)	70% ethanol – 15 mins	MS media; NAA, BAP; coconut water, ascorbic acid	Whitish bud-like sructures (WBLS) are formed within 2 weeks, BAP (70µM) is best for WBLS formation. Maximum shoot (19.70) found in media supplemented with BAP (31.0µM)	Harirah and Khalid, 2006
Sannachenkadal i (AA), Red banana (AAA)	1% labolene – 5 min, running tap water – 20 mins, 0.1% mercuric chloride – 5 mins	MS media; BA, IAA, 2-ip, Kin	Sannachenkadali and Red banana induced maximum multiple shoots (21.57 and 21.60) in media supplemented with 8.9µM BA and 22.2µM BA respectively	Resmi and Nair, 2007
Berangan (AAA), Rasthali (AAB), Nangka (AAB), Abu (ABB)	70 % ethyl alcohol – 5 min	MS media; TDZ, BAP, Kin, 2-ip, Zea	Highest response for all cultivars in media supplemented with BAP (8 mg/l). TDZ and BAP stimulated multiplication to higher extent compared to Kin, 2-ip and Zea. CLB (Cauliflower-like bodies are formed)	Darvari *et al.*, 2010
Virupakshi (AAB), Sirumalai (AAB)	70 % Ethyl alcohol – 30s , 0.1% mercuric chloride - 30s	MS media; BAP, GA; coconut water, casein hydrolysate, proline	15 shoots from a single part of a flower in MS + BAP (5 mg/l) + 15% c.water within 6-8 months	Mahadev *et al.*, 2011
Meitei Hei	70% ethanol – 5 min, 0.2% mercuric chloride – 5 min	MS (Full and half strength); BAP, NAA, Kin, TDZ,	Regeneration of male inflorescence far from apical dome in basal and middle portion of floral axis was successfully obtained	Punyarani *et al.*, 2013

2.2 Evaluation of genetic fidelity of *in vitro* raised banana plantlets

Genetic fidelity is the maintenance of the genetic constitution of a particular clone throughout its life span (Chaterjee and Prakash, 1996). PCR based molecular markers like Randomly Amplified Polymorphic DNA (RAPD) and Inter-Simple Sequence Repeats (ISSR) have particularly been suggested to be useful for confirmation of genetic fidelity in micropropagated plants (Gupta and Varshney, 1999). RAPD is a technique in which short oligonucleotide primers are arbitrarily selected to amplify a set of DNA segments which are randomly distributed throughout the genome. It was first described by Williams *et al*., (1990) as a commonly used molecular marker in genetic diversity studies. ISSR also involves PCR amplification of the region between two closely placed simple repeat sequences that are inversely oriented. They are identified using primers designed from within the repeated region (Zietjiewicz *et al*., 1994). Both markers have been used to detect somaclonal variation in different micropropagated plants as well as genetic diversity among cultivars and germplasm (Howell *et al*., 1994; Bhat and Jarret, 1995; Damasco *et al*., 1996; Wallner *et al*., 1996; Rival *et al*., 1998; Rani *et al*., 2000, 2001; Gimenez *et al*., 2001; Devarumath *et al*., 2002; Martins *et al*., 2003, 2004).

The genetic fidelity of regenerated plants of banana is often questioned since there are frequent reports on the occurrence of somaclonal variations not only in regenerated plants but also in micropropagated ones (Sahijram *et al*., 2003; Martin *et al*., 2006; Ray *et al*., 2006). Micropropagation of banana is a major activity in most of the commercial tissue culture units where high levels of growth regulators are often used to enhance the rate of shoot multiplication (Dhed'a *et al*., 1991; Bhagyalakshmi and Singh, 1995; Bairu *et al*., 2006). Similarly, the holding of shoot cultures for long

periods *in vitro* during micropropagation is known to result in undesirable clonal variability in important commercial crops such as *Anigozanthos viridis* (Turner *et al*., 2001), *Prunus dulcis* (Martins *et al*., 2004), *Foeniculum vulgare* (Bennici *et al*., 2004), and *Chlorophytum arundinaceum* (Lattoo *et al*., 2006). Kulkarni *et al*. (2004) observed differential effect of genome composition and cytokinins on *in vitro* propagation in banana cultivars. While there are several reports on genetic analyses of *in vitro* developed banana plantlets utilizing explants obtained from sucker (shoot tip meristems), rhizhome etc such as in cv. Grand Naine (Borse *et al*., 2011), cv. Bantala, cv. Grand Naine, cv. Patakapura, cv. Robusta (Rout *et al*., 2009), cv. Nanjanagudu Rasabale (Venkatachalam *et al*., 2007), cv. Robusta, cv. Giant Governor, cv. Martaman (Ray *et al*., 2006) there are fewer reports on explants obtained from male flowers such as in cv. Berangan (Harirah and Khalid, 2006).

2.3 Genetic transformation studies in banana

Among the genetic transformation methods available, *Agrobacterium*-mediated transformation has been extensively applied to many plant species, because this method offers several advantages such as the defined integration of transgenes, potentially low copy number, and preferential integration into transcriptional active regions of the chromosome (Koncz *et al*., 1989; Hiei *et al*., 2000). Schell and Van Montagu (1977) at the University of Ghent (Belgium) discovered the gene transfer mechanism between *Agrobacterium* and plants, which resulted in the development of methods to alter *Agrobacterium* into an efficient delivery system for gene engineering in plants. The genus *Agrobacterium* has been divided into a number of species where among them, *A. tumefaciens* causing crown gall disease and *A. rhizogenes* causing hairy root disease (Gelvin, 2003) are the most widely used species in genetic

transformation of plants after disarming of the tumor-inducing (Ti) and root-inducing (Ri) plasmids respectively. *Agrobacterium* genetically transforms its host by transferring a well-defined DNA segment from its Ti or Ri plasmid to the host-cell genome (Gelvin, 1998). Recombinant *Agrobacterium* strains in which the native T-DNA has been replaced with genes of interests, are the most efficient vehicles used today for the introduction of foreign genes into plants and for the production of transgenic plant species (Draper *et al*., 1988).

It is a well-known fact that monocotyledonous plants are more recalcitrant than dicotyledonous plants to *Agrobacterium*-mediated genetic transformation. Hence, major efforts in these monocotyledonous plant species have previously been taken to improve the transformation efficiency through direct gene transfer techniques. However, in the past few years, banana plants have been transformed through *Agrobacterium*-mediated genetic transformation (Ganapathi *et al*., 2001a; May *et al*., 1995; Maziah *et al*., 2007; Huang *et al*., 2007). Currently, most of the transformation protocols for banana are based on cell suspension cultures (Ganapathi *et al*., 2001a; Khanna *et al*., 2004; Becker *et al*., 2000; Sagi *et al*., 1995). There are many reports on banana transformation using different target explants (Table 2.2), while there are only few countable reports on banana transformation using male flower as the target explant. Initiation and maintenance of cell suspension cultures are very difficult and require more time to obtain transgenic banana plants. Hence, it is necessary to develop alternative regeneration and transformation protocols to obtain transgenic banana plants within a short time.

Table 2.2 Review on banana genetic transformation studies.

Cultivar name & genotype	Target explant	Type of organogenesis	Incubation	Strain + Vector	Authors
Rasthali (AAB)	*In vitro* shoot tips	Indirect-ECS	-	EHA105, pVGSUN	Ganapathi *et al*., 2001a
Grand naine (AAA), Lady finger (AAB)	Male flower	Indirect-ECS	-	AGL1 and LBA4404, pCAMBIA 1305.1	Khanna *et al*., 2004
Agbagba (AAB)	Apical shoot tips- *in vitro* raised	Direct	In dark- 3 days	EHA105, pCAMBIA 1201	Tripathi *et al*., 2005
Rasthali (AAB)	*In vitro* shoot tips	Indirect-ECS	3 days	EHA105, harbouring pHBS, pHER, pEFEHBS, and pEFEHER.	Kumar *et al*., 2005
Rasthali (AAB)	*In vitro* corms to give multiple buds	Direct-single buds	In dark, 3 days, 22°C	EHA101, pBI333-EN4-RCC2	Sreeramanan *et al*., 2006
Mas (AA)	Male flower	Indirect-ECS	A) 100 rpm for 72 to 168h. B)40 rpm for 12 h followed by 100 rpm for 60 or 156 h.	EHA105, pCAMBIA2301	Huang *et al*., 2007
Rastali (AAB)	Single buds from *invitro* plantlets	Direct	In dark-3 days, 22°C	EHA 101, pB1333-EN4- RCC2	Maziah *et al*., 2007
East African highland banana (AAA)	Intercalary meristematic tissues (*in vitro*)	Direct	In dark- 3 days	EHA105, pCAMBIA2301	Tripathi *et al*., 2008
Robusta (AAA)	Male flowers	Indirect-embryogenic cell suspensions (ECS)	In dark- intial 12 hrs at 40 rpm, then 3-7 days at 100 rpm	EHA105,pCAMBIA 1301	Ghosh *et al*., 2009
Nanicao (AAA)	Immature fruits	-	-	-	Matsumoto *et al*., 2009
Pei Chiao (AAA), Gros Michel (AAA)	Multiple bud clumps (MBC)	Direct	In dark- 2 days	EHA105 and C58C1	Yip *et al*., 2011
Williams (AAA)	Sucker	Direct – i*nvitro* apical shoot tips, bisected longtudinally, previously incubated in dark at 28°C overnight	100 µM Acetosyringone, in the dark, 3 days, 28°C	EHA105, GV2260 and C58, pCAMBIA 1201	Esuola *et al*., 2011
Grand naine (AAA)	Immature flowers	Indirect-ECS	In dark, 6 days, 21°C	EHA105, pGmhsp-A and pAthsp-A	Chong-Pérez *et al*., 2012

Chapter 3

Characterization of economically important banana varieties of Mizoram

CHAPTER 3

CHARACTERIZATION OF ECONOMICALLY IMPORTANT BANANA VARIETIES OF MIZORAM

3.1 Introduction

Mizoram (21˚58' to 24˚35' N - 92˚15' to 93˚29' E) is located in the region which is regarded as the reservoir for the large genepool of banana genetic resources, and is the meeting point of *M. balbisiana* of the Indian subcontinent and *M. acuminata* of Southeast Asia (Molina and Kudamage, 2002). Numerous seeded and non-seeded banana cultivars are found abundantly growing in the wild as well as in cultivated areas. Altogether fourteen banana cultivars have been identified out of which seven were edible and seven were seeded (Lalrinfela and Thangjam, 2012). Important commercial cultivars are Vaibalhla (*M. acuminata* AAA group), Grand naine (*M. acuminata* AAA group), Banria (*Musa* ABB group) and Lawngbalhla (*Musa* AAB group). The average yield of annual banana production of the state from 2009-2013 was 112501 metric tonnes from an average area of 9832.50 Ha (Department of Horticulture, Government of Mizoram, 2013).

Banana cultivation in Mizoram is mainly carried out by the farmers with small land areas. Based on the cultivar and/or location of the plantation sites the income of the growers varies. The yield of the crops also varies from place to place depending on the agro-climatic conditions. Thus a proper investigation was necessary to study the economic potential of different cultivars grown in different phytogeographical regions of Mizoram and also to evaluate the level of genetic variation among the cultivars.

3.2 Materials and Methods:

3.2.1 Identification of commercially important banana cultivars and their cultivation sites in Mizoram.

For the identification of commercially important banana cultivars grown in Mizoram, an exhaustive market survey was carried out in the main commercial market in Aizawl (Bara Bazar area) during February – April, 2009. Monthly market price of the cultivars was collected for the calculation of the economic value. Economic values were calculated on the basis of rupees per kilogram. The average number of fingers per kilogram of each cultivar was calculated from 10 random samples. The number of fingers per kilogram varies in the ranges of 4-5 in Vaibalhla, 5-8 in Banria and 5-6 in Lawngbalhla respectively. The cost of fingers of each cultivar was consistent and calculated as: Rs. 17/kg (Vaibalhla), Rs. 20/kg (Banria) and Rs. 15/kg (Lawngbalhla). After the identification of the cultivars, the major plantation sites were selected for the study based on the information provided by the local farmers and officials of the state Horticulture department.

After obtaining the information on the distribution of the selected banana cultivars in Mizoram from the concerned officials of the Horticulture department of the state, expeditions to the plantation of the cultivars were carried out during the month of May – August, 2009. Altogether 7 different locations in Mizoram were covered in the present study on the basis of phytogeographical regions (Fig. 3.1). The regions selected for the study are as follows:

I. Tropical:

i. Tanhril, Aizawl District: Longitude: 92°43'3"E, Latitude: 23°43'27"N, Altitude: 801 mASL

ii. Jubilee Veng, Aizawl District: Longitude: 92°42'22.47"E, Latitude:23°42'50.28"N, Altitude: 938 mASL

iii. Tuivamit, Aizawl District: Longitude: 92°40'51"E, Latitude: 23°44'35"N, Altitude: 801 mASL

II. Sub-tropical:

i. Saikhamakawn, Aizawl District: Longitude: 92°43'3"E, Latitude: 23°43'27"N, Altitude: 1132 mASL

ii. Khumtung, Serchhip District: Longitude: 92°51'05.32"E, Latitude: 23°33'26.10"N, Altitude: 1040 mASL

iii. Keifang, Aizawl District: Longitude: 92°57'40.94’’E, Latitude: 23°39'59.03"N, Altitude: 1078 mASL

III. Temperate:

i. Khuaimual, Champhai District: Longitude: 92°43'3"E, Latitude: 23.5°0'00"N, Altitude: 1672 mASL

3.2.2 Evaluation of agronomic characters and plantation sites

The details of the agronomic characters and economic potential of the 3 cultivars grown in different phytogeographical regions of Mizoram were recorded from the plantation sites based on standard methods. The parameters are as follows:

i. Pseudostem height
ii. Leaf colour
iii. Leaf area
iv. Leaf index
v. Number of fingers per bunch
vi. Number of bunch per plant
vii. Finger weight
viii. Yield
ix. Pulp colour at maturity
x. Peel colour at maturity
xi. Gross income per plant

The average data for all the 3 cultivars collected from 7 sites were used for the identification of the cultivar with the highest economic potential. Similarly, the most suitable phytogeographical region for each cultivar was also determined. From the identified banana growing site having the highest agronomic and economic value in Mizoram (Khumtung, Serchhip district) 10 individual plants of unknown origin for each cultivar were identified and tagged. From each of the identified individual plants 2-3 sword suckers were randomly collected, labeled and then transported for maintenance in the Field Gene Bank of the Department of Biotechnology, Mizoram University. The suckers were planted in a cluster with 5 × 5 ft spacing in rows in the field gene bank under the same environmental condition for continuous evaluation and further analysis.

3.2.3 Evaluation of genetic variations among the cultivars using RAPD markers

3.2.3.1 DNA extraction: Young cigar leaves from each of the 10 individual plants representing the 3 cultivars (Vaibalhla, Banria and Lawngbalhla) were collected from the Field Gene Bank of the Department of Biotechnology, Mizoram University and were used for DNA isolation. The genomic DNA from cigar leaves (100 mg) was isolated by the modified CTAB method (Thangjam *et al.*, 2003) with slight modifications as described below.

Protocol for DNA isolation:

1. Collect about 100 mg of leaves from each cultivar, rinse with tap water and blot dry.

2. Place the cultivar in the micro-centrifuge tube (2 mL) containing 400 μL of extraction buffer.

3. Grind the leaves in the buffer inside the tube with sterile glass rod.

4. Incubate at 37°C for at least 90 min in water bath.

5. Add 400 µL of 2% CTAB solution and incubate at 65°C at least for 120 min.

6. Cool to room temperature, then extract by gently adding equal amount of chloroform: isoamyl alcohol (24:1) containing 5% phenol.

7. Centrifuge at 12,000 rpm in a microcentrifuge at 4°C for 5 min.

8. Carefully transfer the upper aqueous layer to a new tube and repeat the extraction process 3 times to remove the cloudiness of the upper layer.

9. Add 2/3rd volume of ice cold isopropanol, mix gently by repeated inversions and incubate at room temperature for 30 min to precipitate DNA.

10. Centrifuge at 12,000 rpm for 5 min at 4°C and remove the supernatant.

11. Wash the pellet with ice cold 70% ethanol.

12. Decant the supernatant and air-dry the pellet.

13. Resuspend the pellet in 100 µL of TE buffer and remove RNA by adding 1 µL of RNase (50 µg/mL).

14. Incubate at 37°C for approximately 60 min (An overnight RNAse treatment helped achieving in proper genomic DNA)

3.2.3.2 Quantification and estimation of DNA quality: The amount of isolated DNA per milligram of leaf tissue was estimated by measuring absorbance at 260 nm and

280 nm using Bio-photometer plus (Eppendorf, Germany) according to the manufacturer's instruction. The purity of DNA was determined by calculating the ratio of absorbance at 260 nm to that of 280 nm. A 100 ng/µL DNA stock was prepared from the isolated DNA to be used for further experiments. The isolated DNA from each of the cultivars was separated on 0.8% agarose gel in 1× TBE buffer. For this, aliquot of 10 µL (1 µg) from 100 ng/µL DNA stock was loaded into the gel and then photographed using a gel documentation system (Bio-rad, Australia). For PCR amplification a DNA stock of 10 ng/µL for each of the cultivars were prepared.

3.2.3.3 RAPD-PCR amplification: RAPD-PCR amplifications were carried out with 10 RAPD primers (UBC set #5) obtained from University of British Columbia (UBC, Vancouver, Canada) and performed in a Thermal Cycler (Bio Rad, C1000™) with the following conditions: 3 min at 94°C; 35 cycles of: 1 min at 94°C, 1 min at 36°C, 2 min at 72°C and a final extension step 10 min at 72°C. The PCR reactions were carried out in a 20 µl reaction mixture volume containing 50 ng of template DNA, 100 µM dNTP mix (Himedia, India), 0.6 µM of random primers, 1× Taq DNA polymerase buffer containing 15mM $MgCl_2$ (Sigma-Aldrich Pvt. Ltd., Bangalore) and 1.0 U of Taq polymerase (Sigma-Aldrich). The amplified products were resolved by electrophoresis on a 1.2% (w/v) agarose gels run in 1× TBE buffer and detected by ethidium bromide staining (Sambrook *et al*., 1989). A 100 bp DNA ladder (Bangalore Genei, India) was used as marker. The resulting fragments were scored under UV light using a gel documentation system (Bio-rad, Australia) and photographed. All the PCR reactions were repeated at least twice to check the reproducibility. Only the distinct, reproducible, and well-resolved fragments were scored as present (+) or absent (-).

3.2.3.4 Cluster analysis

Rectangular binary data matrix of RAPD was used for statistical analyses. Pairwise dissimilarity matrix was generated using Nei's coefficient (Nei, 1978). A dendrogram using neighbour joining method (unweighted) was constructed on the basis of the dissimilarity matrix data. All the analysis was conducted using the software NTSYS-pc version 2.20a (Rohlf, 2000).

3.3 Results

3.3.1 Identification of commercially important banana cultivars

The result of the exhaustive market survey conducted during February – April, 2009 to identify the commercially important banana cultivars in Mizoram revealed 3 cultivars i.e. Vaibalhla (*M. acuminata* AAA), Banria (*Musa* ABB) and Lawngbalhla (*Musa* AAB) (Fig. 3.2). The economic value of the bananas in the market was determined from the cost/kg (no. of fingers/kg/Rs.). The average number of fingers per kilogram was 4.5 in Vaibalhla, 6.5 in Banria and 5.5 in Lawngbalhla. The market value of the cultivars was Rs. 17/kg for Vaibalha, Rs. 20/kg for Banria and Rs. 15/kg for Lawngbalhla respectively. The economic value of the banana cultivars (Rs. /finger) was in order of Vaibalhla (Rs. 3.77) > Banria (Rs. 3.07) > Lawngbalhla (Rs. 2.72) (Table 3.5 and Fig. 3.6).

3.3.2 Evaluation of cultivars and collection site

The distributions of all the 3 cultivars were surveyed in selected 7 cultivation sites (Table 3.1). Banria and Lawngbalhla were found in all the sites except cv. Vaibalhla, which was not found in the temperate regions. The mean value of agronomic

characters and economic potential viz. pseudostem height, leaf colour, leaf area, leaf index, number of fingers per bunch, finger weight, number of bunch per plant, yield, pulp colour at maturity, peel colour at maturity and gross income per plant were calculated from 10 samples of each cultivar in the 7 collection sites. Details of the evaluated agronomic characters and economic potential for Vaibalhla, Banria and Lawngbalhla from the different phytogeographical regions are provided in Table 3.2, Table 3.3 and Table 3.4 respectively. A comparison was made among the 3 cultivars by taking an average from the agronomic and economic potential data from all 7 locations showed that Vaibalhla had the highest economic potential (Rs. 400.30 gross income/plant) as shown in Table 3.6 and Figure 3.7. The average agronomic and economic value of the cultivars from the different phytogeographical regions also showed variable values. For Vaibalhla (Table 3.7 and Fig. 3.8) the pseudostem height was in order of sub-tropical (266.6 cm) > tropical (234.89 cm), leaf length was in order of tropical (237.3 cm) > sub-tropical (235.56 cm), leaf width was in order of tropical (74.40 cm) > sub-tropical (73.06 cm), leaf index was in order of sub-tropical (3.22) > tropical (3.18), number of fingers per bunch- was in order of sub-tropical (14.23) > tropical (13.83), the order of finger weight was tropical (0.21 kg) > sub-tropical (0.20 kg), the number of bunch per plant was in order of tropical (7.63) > sub-tropical (7.46), yield was sub-tropical (25.74 kg/plant) > tropical (21.41 kg/plant) and the gross income per plant was sub-tropical (Rs. 437.65/plant) > tropical (Rs. 364.01/plant) respectively.

Similarly, the agronomic and economic values for Banria (Table 3.8 and Fig. 3.9) were evaluated. The pseudostem height was in the order of temperate (460.8 cm) > tropical (450.63 cm) > sub-tropical (450.26 cm), leaf length was in the order of sub-

tropical (190.10 cm) > tropical (188.53 cm) > temperate (188.30 cm), leaf width was in the order of sub-tropical (55.90 cm) > tropical (50.40 cm)> temperate (48.10 cm), leaf index was in the order of temperate (3.91) > tropical (3.73) > sub-tropical (3.41), the order of number of fingers per bunch was tropical (16.83) > temperate (16.50) > sub-tropical (16.46), the order of finger weight was in the order of sub-tropical (0.15 kg) > temperate (0.14 kg) > tropical (0.13 kg), the number of bunch per plant was in the order of temperate (7.70) = sub-tropical (7.70) > tropical (7.26), the yield was in the order of sub-tropical (19.36 kg/plant) > temperate (17.8 kg/plant) > tropical (16.83 kg/plant) and the gross income per plant was in the order of sub-tropical (Rs.388.40/plant) > temperate (Rs. 356.00/plant) > tropical (Rs. 336.65/plant).

For Lawngbalhla (Table 3.9 and Fig. 3.10) the pseudostem height was in the order of sub-tropical (243.8 cm) > tropical (236.49 cm) > temperate (232.56 cm), the leaf length was in the order of tropical (128.2 cm) > sub-tropical (126.8 cm) > temperate (124.3 cm), the leaf width was in the order of tropical (56.86 cm) > sub-tropical (55.98 cm)> temperate (53.5 cm), the order of leaf index was temperate (2.32) > sub-tropical (2.26) > tropical (2.25), the order of number of fingers per bunch was sub-tropical (14.46) > temperate (14.20) > tropical (13.63), the order of finger weight was in the order of sub-tropical (0.18 kg) = temperate (0.18 kg) > tropical (0.17 kg), the number of bunch per plant was in the order of temperate (10.90) = sub-tropical (10.70) > tropical (10.06), the order of yield was sub-tropical (28.35 kg/plant) > temperate (27.91 kg/plant) > tropical (24.29 kg/plant) and the order of gross income per plant was sub-tropical (Rs. 424.33/plant) > temperate (Rs.418.77/plant) > tropical (Rs.364.45/plant).

It was also observed that the sub-tropical regions were more suitable for all the 3 cultivars based on the economic values. Thus, Khumtung area in Serchhip District, a sub-tropical zone was selected for the collection of plants.

3.3.3 RAPD analysis

10 RAPD primers (UBC set #5) obtained from University of British Columbia, which produced clear and reproducible banding pattern in all the cultivars were used for the experiment. The random primers used were UBC-416, UBC-418, UBC-419, UBC-420, UBC-421, UBC-433, UBC-434, UBC-440, UBC-449 and UBC-450 (Table 3.10). The result of the RAPD-PCR profiles of the 10 individual plants of each cultivar showed monomorphic bands (Fig. 3.11 and Fig. 3.12). Among the primers, UBC-420 produced the maximum number of bands (15) (Fig. 3.11 D) while the minimum was found with UBC-440 primer (6) (Fig. 3.12 C). The resulting dendrogram calculated from the RAPD profiles generated by the samples using 10 primers clearly showed the 3 groups. All the individual plants of each cultivar were clustered together in the same group (Fig. 3.13).

3.4 Discussion

The economic survey for the identification of the potential banana cultivars native to Mizoram were identified as Vaibalhla (AAA), Banria (ABB) and Lawngbalhla (AAB). The most popular cultivar with the highest economic value was Vaibalhla. This cultivar is a triploid *M. acuminata* (AAA) of the Cavendish sub-group having a sweet taste (Lalrinfela and Thangjam, 2012). It has been reported by Lescot (2011) that half of the current banana production relies on somaclones derived Cavendish dessert bananas (AAA group). The world export of banana in 2007, consisted mainly

of Cavendish banana, which was estimated to be 18 million tons (20% of world production), amounting to US$ 7.2 billion in economic terms (FAOSTAT, 2009). The most important attributes that make the Cavendish sub-group the main bananas for export are related to their reliability during transport and their shelf life. Thus, further studies on the Cavendish cultivar Vaibalhla based on *in vitro* mass propagation and genetic transformation could result in greater economic potential for the state of Mizoram. Among the 3 cultivars identified, 2 (Banria and Lawngbalhla) were found throughout the state while Vaibalhla was found only in the tropics. It is a clear known fact that banana (*M. acuminata*) having the A genome are more cold-sensitive as compared to plantains (*M. balbisiana*) having the B genome, even though the physiological mechanisms of cold-tolerance for plantains are still not clear (Zhang *et al*., 2011). This is in agreement with the present study finding where Vaibalhla (AAA) was not found in the temperate region of Mizoram (Champhai district), while Banria (ABB) and Lawngbalhla (AAB) thrives well in all the phytogeographical regions including the temperate regions.

The genetic monomorphism detected among the individuals within the 3 cultivars studied (Vaibalhla, Banria and Lawngbalhla) collected from Khumtung, Serchhip district showed that the plants were genetically close. Banana multiplication is mostly through the suckers by means of vegetative propagation (Ganapathi *et al*., 2001b) since viable seeds are not produced in edible triploid bananas. Premabati *et al*. (2013) analyzed 7 edible cultivars of Mizoram belonging to different genome groups showed a significant amount of genetic diversity using RAPD markers. Similarly, in the present study genetic variations among the cultivars were detected, as revealed by the dendrogram.

Table 3.1 Details of the distribution of economically important banana cultivars in different phytogeographical regions of Mizoram.

Cultivar Name	Scientific name	**Phytogeographical region**						
		Tropical			Sub-tropical			Temperate
		Tanhril, Aizawl District	Jubliee Veng, Aizawl District	Tuivamit, Aizawl District	Saikhamakawn, Aizawl District	Khumtung, Serchhip District	Keifang, Aizawl District	Khuaimual, Champhai District
		Long: 92°43'3"E Lat: 23°43'27"N Alt: 801 mASL	Long:92°42'22.47"E Lat:23°42'50.28"N Alt: 938 mASL	Long:92°40'51"E Lat:23°44'35"N Alt: 801 mASL	Long:92°43'3"E Lat:23°43'27"N Alt: 1132 mASL	Longe:92°51'05.32"E Lat:23°33'26.10"N Alt: 1040 mASL	Long:92°57'40.94"E Lat:23°39'59.03"N Alt: 1078 mASL	Long:92°43'3"E Lat:23.5°0'00"N Alt: 1672 mASL
Vaibalhla	*Musa acuminata* AAA	+	+	+	+	+	+	-
Banria	*Musa* sp. ABB	+	+	+	+	+	+	+
Lawngbalhla	*Musa* sp. AAB	+	+	+	+	+	+	+

+ presence; - absence ; Long: Longtitude; Lat: Latitude; Alt: Altitude

Table 3.2 Agronomic characters and economic potential of *M. acuminata* cv. Vaibalhla (AAA) collected grown in different phytogeographical regions of Mizoram.

Site	Pseudostem height (cm)	Leaf colour	Leaf area		Leaf index	No. of fingers/ bunch	Finger weight (kg)	No. of bunch/ plant	Yield (kg/plant)	Pulp colour at maturity	Peel colour at maturity	Gross Income (Rs/ plant) calculated from the average of Rs. 17/kg	Symptoms of disease
			Length (cm)	Width (cm)									
Tuivamit, Aizawl District (Tropical)	232.59 ± 0.33	dark green	240.30 ± 0.24	74.52 ± 0.23	3.22 ± 0.04	13.50 ± 0.30	0.21 ± 0.003	7.20 ± 0.20	20.37 ± 0.71	yellow	yellow	346.44 ± 12.21	Nil
Jubilee Veng, Aizawl District (Tropical)	228.30 ± 0.18	dark green	232.20 ± 0.20	73.20 ± 0.26	3.17 ± 0.03	14.20 ± 0.24	0.22 ± 0.002	7.70 ± 0.26	24.10 ± 1.10	yellow	yellow	409.76 ± 18.74	Nil
Tanhril, Aizawl District (Tropical)	243.80 ± 0.24	dark green	239.4 ± 0.12	75.50 ± 0.33	3.17 ± 0.03	13.80 ± 0.29	0.19 ± 0.004	7.50 ± 0.16	19.75 ± 0.99	yellow	yellow	335.83 ± 16.99	Nil
Saikhamakawn, Aizawl District (Sub-tropical)	235.20 ± 0.49	dark green	237.70 ± 0.37	75.10 ± 0.42	3.16 ± 0.03	13.70 ± 0.26	0.21 ± 0.005	7.50 ± 0.16	21.67 ± 1.10	yellow	yellow	368.52 ± 18.73	Nil
Khumtung, Serchhip District (Sub-tropical)	243.30 ± 0.32	dark green	234.50 ± 0.25	71.80 ± 0.47	3.26 ± 0.04	15.0 ± 0.25	0.25 ± 0.003	8.10 ± 0.23	30.46 ± 1.35	yellow	yellow	517.85 ± 22.97	Nil
Keifang, Aizawl District (Sub-tropical))	231.50 ± 0.31	dark green	234.50 ± 0.23	72.30 ± 0.27	3.24 ± 0.03	14.0 ± 0.25	0.23 ± 0.002	7.80 ± 0.13	25.09 ± 0.58	yellow	yellow	426.58 ± 9.94	Nil
Khuaimual, Champhai District (Temperate)	-	-	-	-	-	-	-	-	-	-	-	-	-

Data represented were mean of 10 individual plants.

Table 3.3 Agronomic characters and economic potential of *Musa sp* cv. Banria (ABB) grown in different phytogeographical regions of Mizoram.

Site	Pseudostem height (cm)	Leaf colour	Leaf area		Leaf index	No. of fingers/ bunch	Finger weight (kg)	No. of bunch/ plant	Yield (kg/plant)	Pulp colour at maturity	Peel colour at maturity	Gross Income (Rs/ plant) calculated from the average of Rs. 20/kg	Symptoms of disease
			Length (cm)	Width (cm)									
Tuivamit, Aizawl District (Tropical)	451.9 ± 0.34	dark green	187.30 ± 0.22	51.50 ± 0.23	3.63 ± 0.02	16.30 ± 0.33	0.14 ± 0.007	6.80 ± 0.24	15.49 ± 1.35	cream	yellow	313.82 ± 27.00	Nil
Jubliee Veng, Aizawl District (Tropical)	430.30 ± 0.36	dark green	187.30 ± 0.22	49.50 ± 0.16	3.76 ± 0.03	17.00 ± 0.29	0.14 ± 0.002	7.20 ± 0.24	17.23 ± 0.97	cream	yellow	344.74 ± 19.48	Nil
Tanhril, Aizawl District (Tropical)	469.70 ± 0.28	dark green	191.00 ± 0.36	50.20 ± 0.21	3.80 ± 0.02	17.20 ± 0.24	0.13 ± 0.003	7.80 ± 0.35	17.57 ± 1.24	cream	yellow	351.40 ± 24.87	Nil
Saikhamakawn, Aizawl District (Sub-tropical)	462.10 ± 0.35	dark green	189.30 ± 0.31	57.50 ± 0.33	3.29 ± 0.03	16.80 ± 0.20	0.12 ± 0.002	8.10 ± 0.23	16.41 ± 0.81	cream	yellow	331.80 ± 16.69	Nil
Khumtung, Serchhip District (Sub-tropical)	431.50 ± 0.36	dark green	190.40 ± 0.30	50.30 ± 0.15	3.78 ± 0.03	17.10 ± 0.27	0.18 ± 0.003	7.50 ± 0.16	23.05 ± 0.66	cream	yellow	461.10 ± 13.32	Nil
Keifang, Aizawl District (Sub-tropical)	457.20 ± 0.23	dark green	190.60 ± 0.27	59.90 ± 0.14	3.18 ± 0.04	15.50 ± 0.30	0.16 ± 0.004	7.50 ± 0.26	18.61 ± 0.90	cream	yellow	372.32 ± 18.04	Nil
Khuaimual, Champhai District (Temperate)	460 .80 ± 0.31	dark green	188.30 ± 0.39	48.10 ± 0.21	3.91 ± 0.06	16.50 ± 0.37	0.14 ± 0.002	7.70 ± 0.30	17.80 ± 0.88	cream	yellow	356.00 ± 17.72	Nil

Data represented were mean of 10 individual plants**.**

Table 3.4 Agronomic characters and economic potential of *Musa sp* cv. Lawngbalhla (AAB) grown in different phytogeographical regions of Mizoram.

Collection site	Pseudostem height (cm)	Leaf colour	Leaf area		Leaf index	No. of fingers/ bunch	Finger weight (kg)	No. of bunch/ plant	Yield (kg/plant)	Pulp colour at maturit y	Peel colour at maturity	Gross Income (Rs/ plant) calculated from the average of Rs. 15/kg	Symptoms of disease
			Length (cm)	Width (cm)									
Tuivamit, Aizawl District (Tropical)	245.56 ± 0.31	dark green	128.20 ± 0.21	57.10 ± 0.29	2.24 ± 0.02	13.30 ± 0.30	0.17 ± 0.002	9.30 ± 0.21	20.98 ± 0.64	cream	pale green	314.84 ± 9.65	Nil
Jubliee Veng, Aizawl District (Tropical)	240.25 ± 0.30	dark green	127.30 ± 0.30	56.50 ± 0.30	2.25 ± 0.03	14.20 ± 0.35	0.17 ± 0.002	10.30 ± 0.21	24.93 ± 1.08	cream	pale green	374.02 ± 16.20	Nil
Tanhril, Aizawl District (Tropical)	223.67 ± 0.32	dark green	129.10 ± 0.23	57.00 ± 0.24	2.26 ± 0.03	13.40 ± 0.30	0.19 ± 0.003	10.60 ± 0.22	26.96 ± 0.90	cream	pale green	404.50 ± 13.59	Nil
Saikhamakawn, Aizawl District (Sub-tropical)	256.90 ± 0.28	dark green	126.40 ± 0.31	56.34 ± 0.23	2.25 ± 0.03	14.50 ± 0.30	0.20 ± 0.004	10.00 ± 0.25	29.07 ± 1.32	cream	pale green	436.06 ± 19.90	Nil
Khumtung, Serchhip District (Sub-tropical)	221.67 ± 0.32	dark green	127.30 ± 0.30	56.90 ± 0.24	2.23 ± 0.03	15.30 ± 0.26	0.18 ± 0.002	11.10 ± 0.31	30.37 ± 1.08	cream	pale green	455.69 ± 16.23	Nil
Keifang, Aizawl District (Sub-tropical))	253.40 ± 0.37	dark green	126.70 ± 0.27	54.70 ± 0.23	2.31 ± 0.03	13.60 ± 0.33	0.17 ± 0.002	11.00 ± 0.25	25.61 ± 0.91	cream	pale green	381.26 ± 13.73	Nil
Khuaimual, Champhai District (Temperate)	232.56 ± 0.35	dark green	124.30 ± 0.24	53.50 ± 0.27	2.32 ± 0.03	14.20 ± 0.24	0.18 ± 0.002	10.90 ± 0.37	27.91 ± 1.30	cream	pale green	418.77 ± 19.64	Nil

Data represented were mean of 10 individual plants.

Table 3.5 Average economic values of the 3 important banana cultivars of Mizoram surveyed during 2009 (February – April)

Cultivar	No. of fingers/ kg	Cost/kg (Rs.)	Cost/finger (Rs.)
Vaibalhla	4.5	17	3.77
Banria	6.5	20	3.07
Lawngbalhla	5.5	15	2.72

Table 3.6 Comparative agronomic and economic potential of three important commercial banana cultivars in Mizoram.

Cultivars	Pseudostem height (cm)	Leaf length (cm)	Leaf width (cm)	Leaf index	No. of fingers/bunch	Finger weight (kg)	No. of bunch/plant	Yield (kg/plant)	Gross income (Rs./plant)
Vaibalhla (AAA)	235.78	236.43	73.73	3.2	14.03	0.21	7.63	23.57	400.83
Banria (ABB)	451.92	189.17	52.42	3.62	16.62	0.14	7.51	18.05	361.59
Lawngbalhla (AAB)	239.14	127.04	56	2.26	14.07	0.18	10.45	26.54	397.87

Table 3.7 Comparative agronomic and economic potential of cv. Vaibalhla (*M. acuminata* AAA) grown in different phytogeographical regions in Mizoram.

Phytogeographical regions	Pseudostem height (cm)	Leaf length (cm)	Leaf width (cm)	Leaf index	No. of fingers/bunch	Finger weight (kg)	No. of bunch/plant	Yield (kg/plant)	Gross income (Rs./plant)
Tropical	234.89	237.30	74.40	3.18	13.83	0.21	7.63	21.41	364.01
Sub-tropical	236.66	235.56	73.06	3.22	14.23	0.20	7.46	25.74	437.65
Temperate	0	0	0	0	0	0	0	0	0

Table 3.8 Comparative agronomic and economic potential of cv. Banria (*Musa* sp. ABB) grown in different phytogeographical regions in Mizoram.

Phytogeographical regions	Pseudostem height (cm)	Leaf length (cm)	Leaf width (cm)	Leaf index	No. of fingers/bunch	Finger weight (kg)	No. of bunch/plant	Yield (kg/plant)	Gross income (Rs./plant)
Tropical	450.63	188.53	50.40	3.73	16.83	0.13	7.26	16.83	336.65
Sub-tropical	450.26	190.10	55.90	3.41	16.46	0.15	7.70	19.36	388.40
Temperate	460.8	188.3	48.10	3.91	16.50	0.14	7.70	17.80	356.00

Table 3.9 Comparative agronomic and economic potential of cv. Lawngbalhla (*Musa* sp. AAB) grown in different phytogeographical regions in Mizoram.

Phytogeographical regions	Pseudostem height (cm)	Leaf length (cm)	Leaf width (cm)	Leaf index	No. of fingers/bunch	Finger weight (kg)	No. of bunch/plant	Yield (kg/plant)	Gross income (Rs./plant)
Tropical	236.49	128.2	56.86	2.25	13.63	0.17	10.06	24.29	364.45
Sub-tropical	243.80	126.8	55.98	2.26	14.46	0.18	10.70	28.35	424.33
Temperate	232.56	124.3	53.50	2.32	14.20	0.18	10.90	27.91	418.77

Table 3.10 Details of the RAPD primers used to in the present study for ascertaining the genetic diversity of three cultivars Vaibalhla, Banria and Lawngbalhla collected from Khumtung area, Serchhip district.

Sl. No	RAPD Primer code	Primer Sequence (5'-3')	Annealing temperature (°C)	Total no. of bands amplified
1	UBC-416	GTGTTTCCGC	36°C	12
2	UBC-418	GAGGAAGCTT	36°C	11
3	UBC-419	TACGTGCCCG	36°C	13
4	UBC-420	GCAGGGTTCG	36°C	15
5	UBC-421	ACGGCCCACC	36°C	14
6	UBC-433	TCACGTGCCT	36°C	7
7	UBC-434	TCGCTAGTCC	36°C	10
8	UBC-440	CTGTCGAACC	36°C	6
9	UBC-449	GAGGTTCAAC	36°C	8
10	UBC-450	CGGAGAGCCA	36°C	13

Figure 3.1 Map of Mizoram showing the cultivation sites of three economically important banana cultivars viz. Vaibalhla, Banria, Lawngbalhla from different phytogeographical regions.

Musa acuminata cv. Vaibalhla (AAA)

Musa sp. cv. Banria (ABB)

Musa sp. cv. Lawngbalhla (AAB)

Figure 3.2 Morphology of three economically important banana varieties of Mizoram.

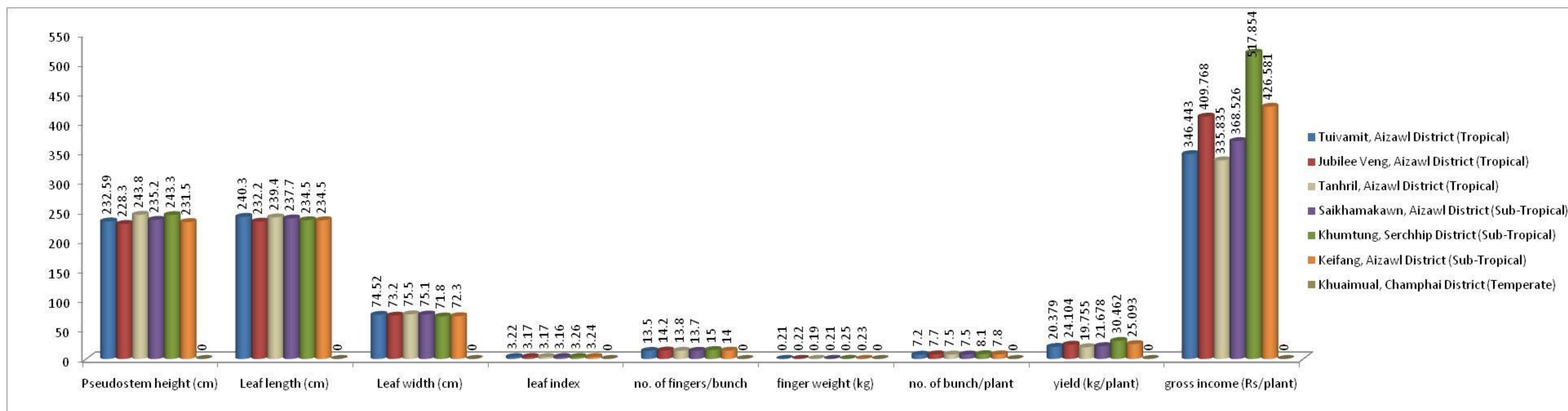

Figure 3.3 Details of the agronomic characters and economic potential of cv. Vaibalhla (*M. acuminata* AAA) grown in different phytogeographical regions of Mizoram.

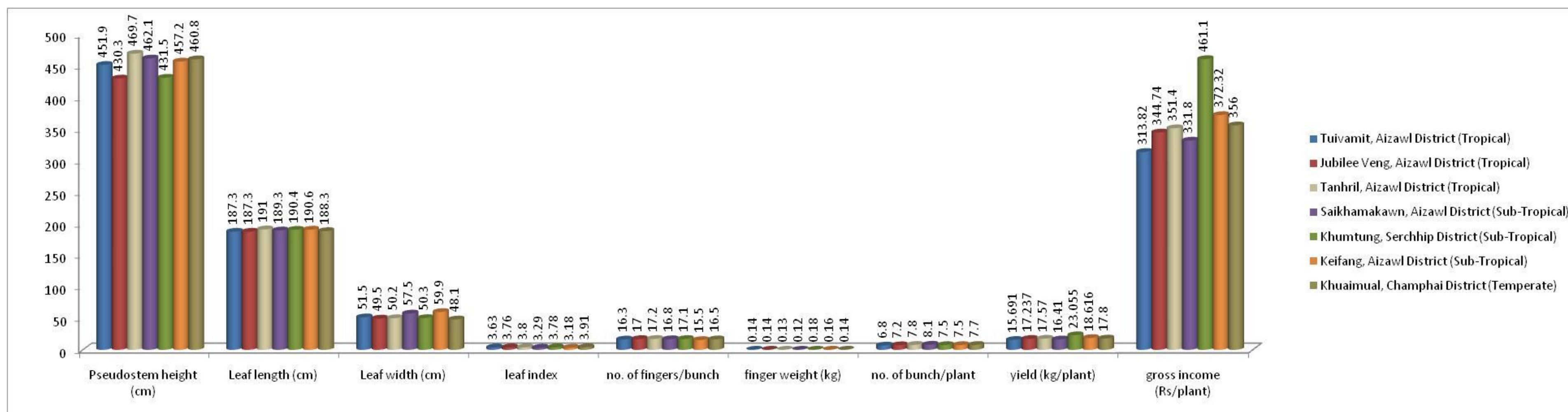

Figure 3.4 Details of the agronomic characters and economic potential of cv. Banria (*Musa* sp. ABB) grown in different phytogeographical regions of Mizoram.

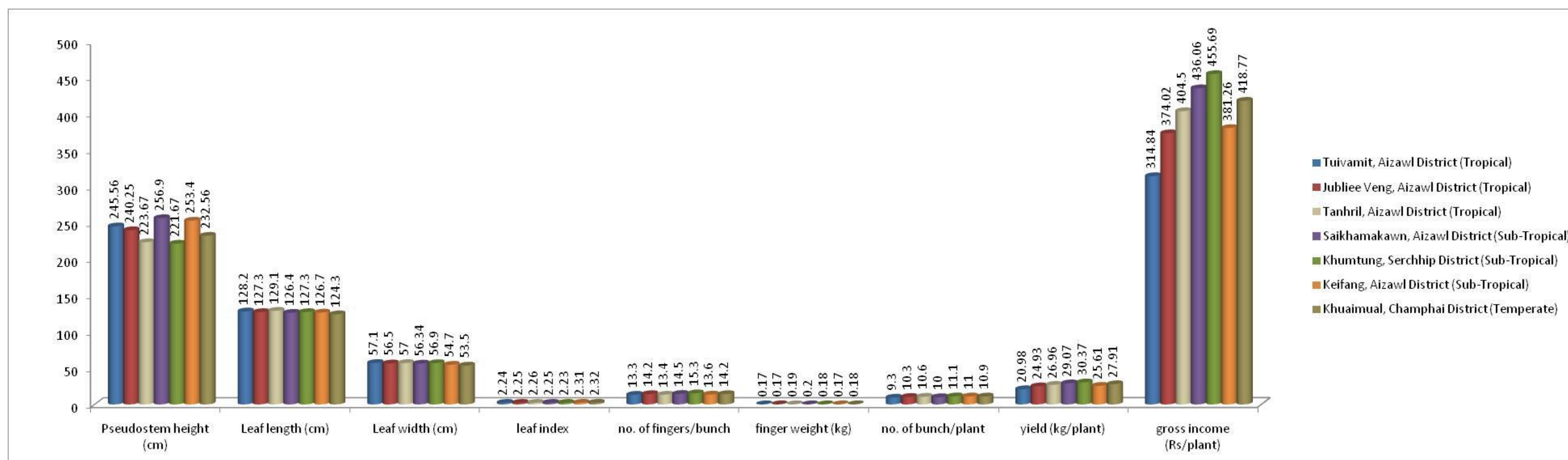

Figure 3.5 Details of the agronomic characters and economic potential of cv. Lawngbalhla (*Musa* sp. AAB) grown in different phytogeographical regions of Mizoram.

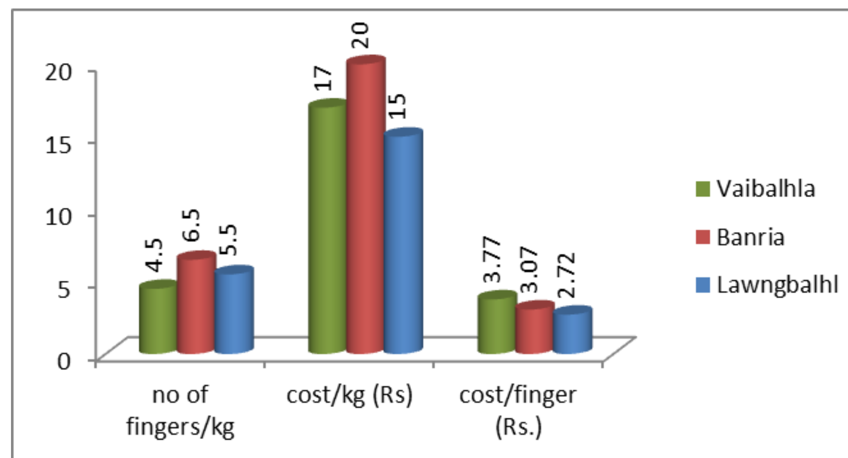

Figure 3.6 Average economic value of the three economically important banana cultivars of Mizoram surveyed during 2009 (February – April).

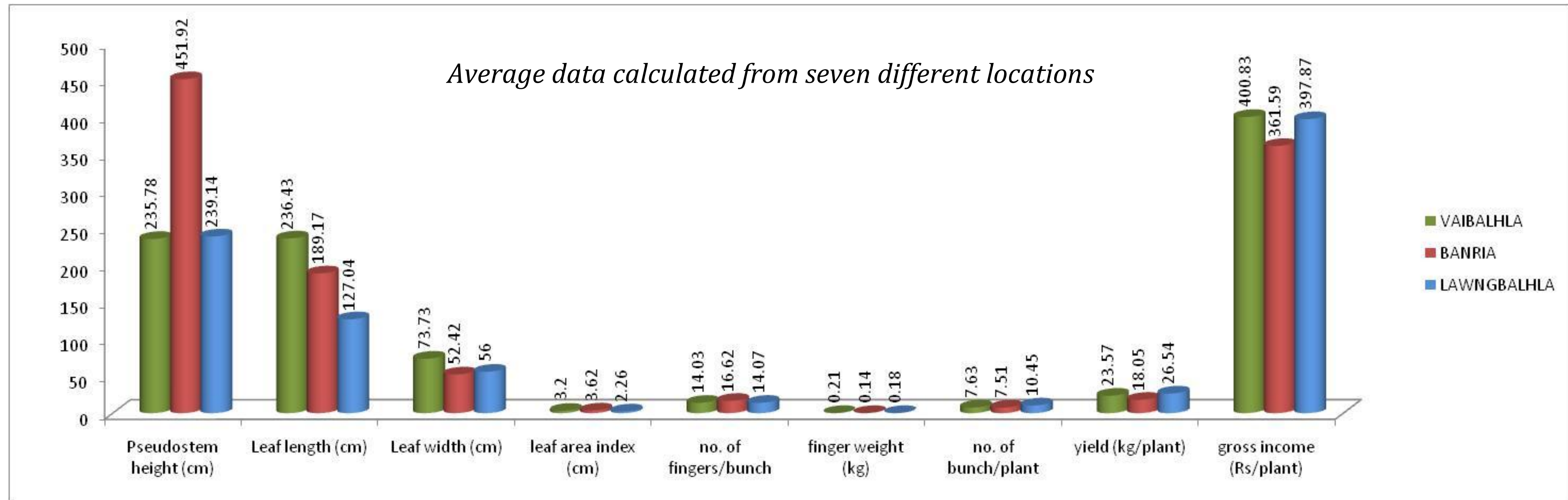

Figure 3.7 Comparative agronomic and economic potential of three important commercial banana cultivars in Mizoram.

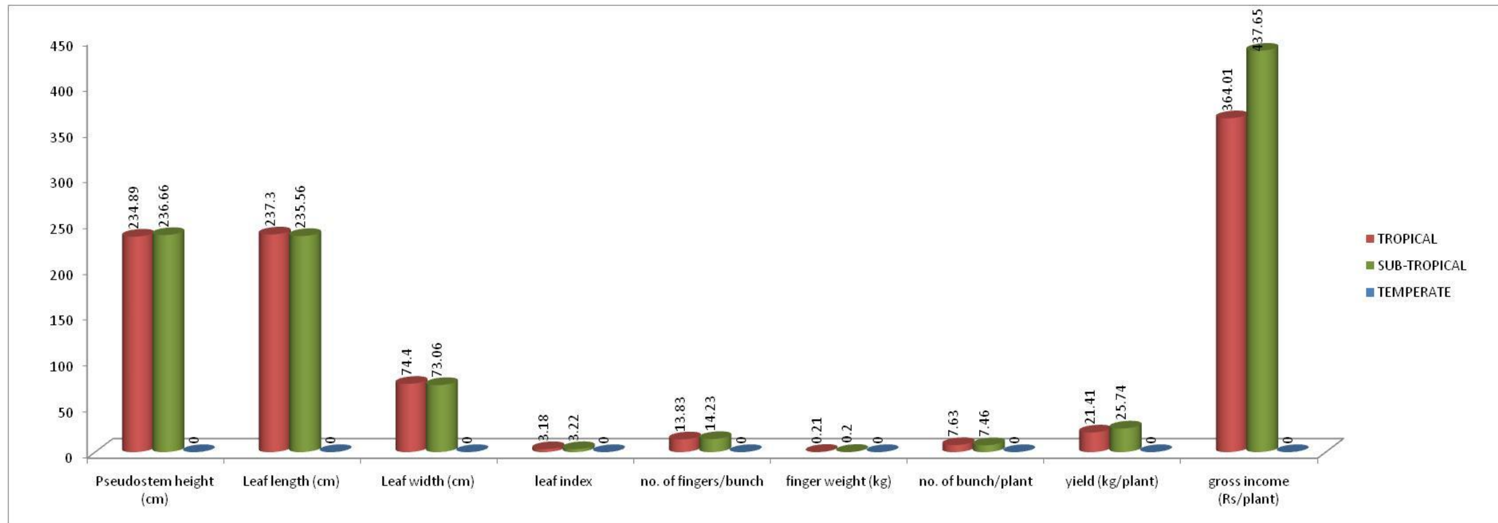

Figure 3.8 Comparative agronomic and economic potential of cv. Vaibalhla (*M. acuminata* AAA) grown in different phytogeographical regions in Mizoram.

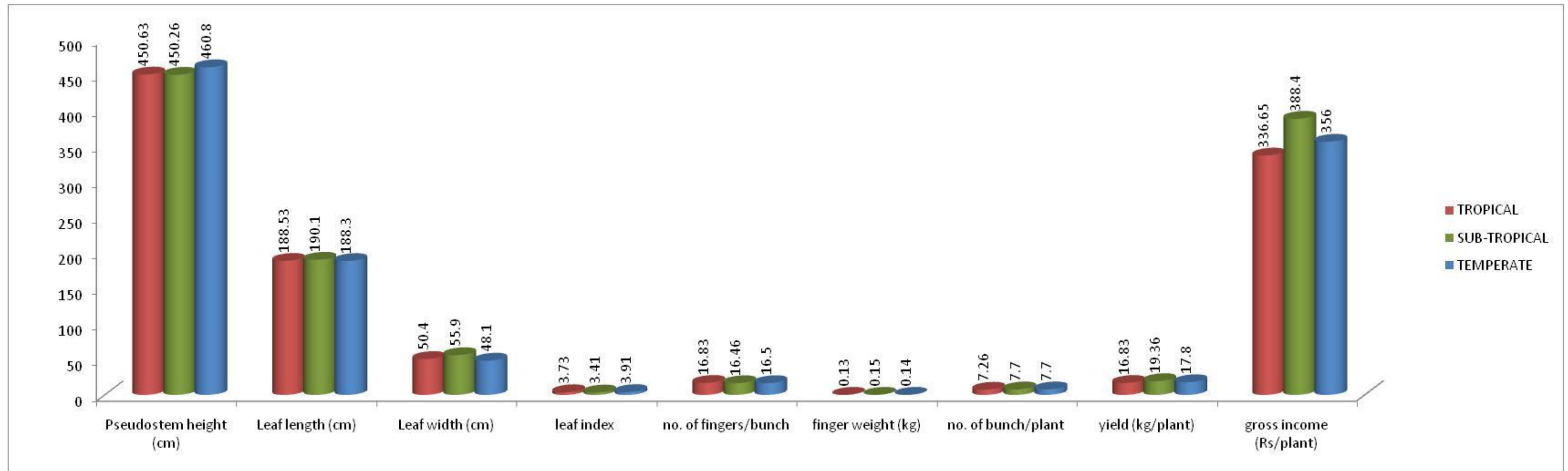

Figure 3.9 Comparative agronomic and economic potential of cv. Banria (*Musa* sp. ABB) grown in different phytogeographical regions in Mizoram.

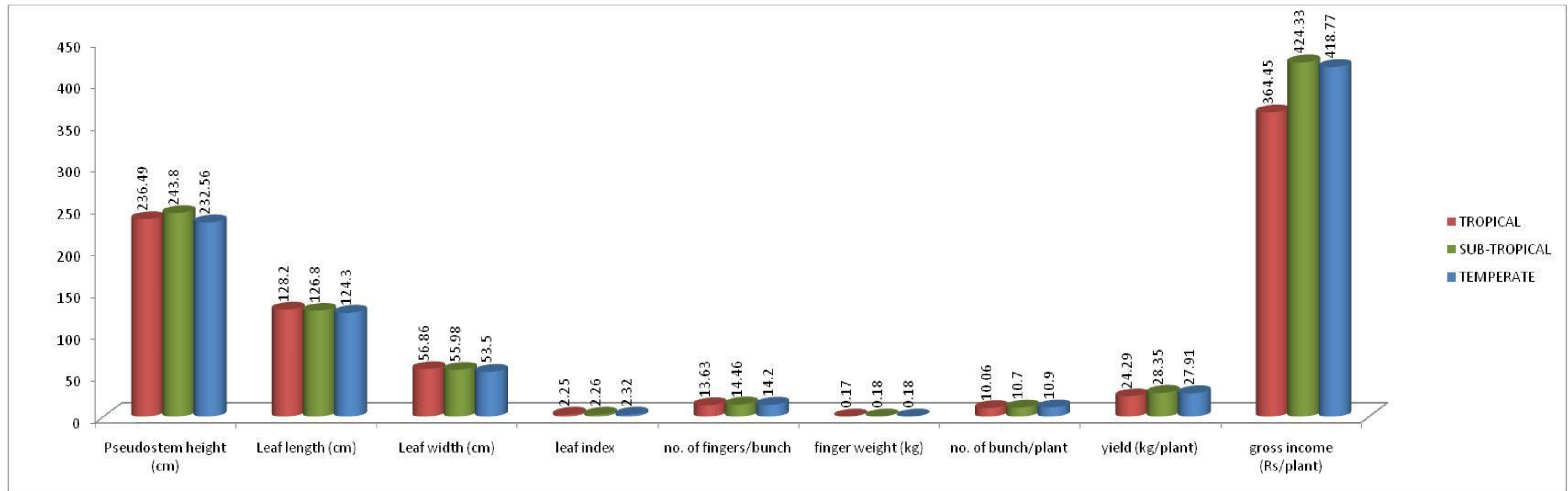

Figure 3.10 Comparative agronomic and economic potential of cv. Lawngbalhla (*Musa* sp. AAB) grown in different phytogeographical regions in Mizoram.

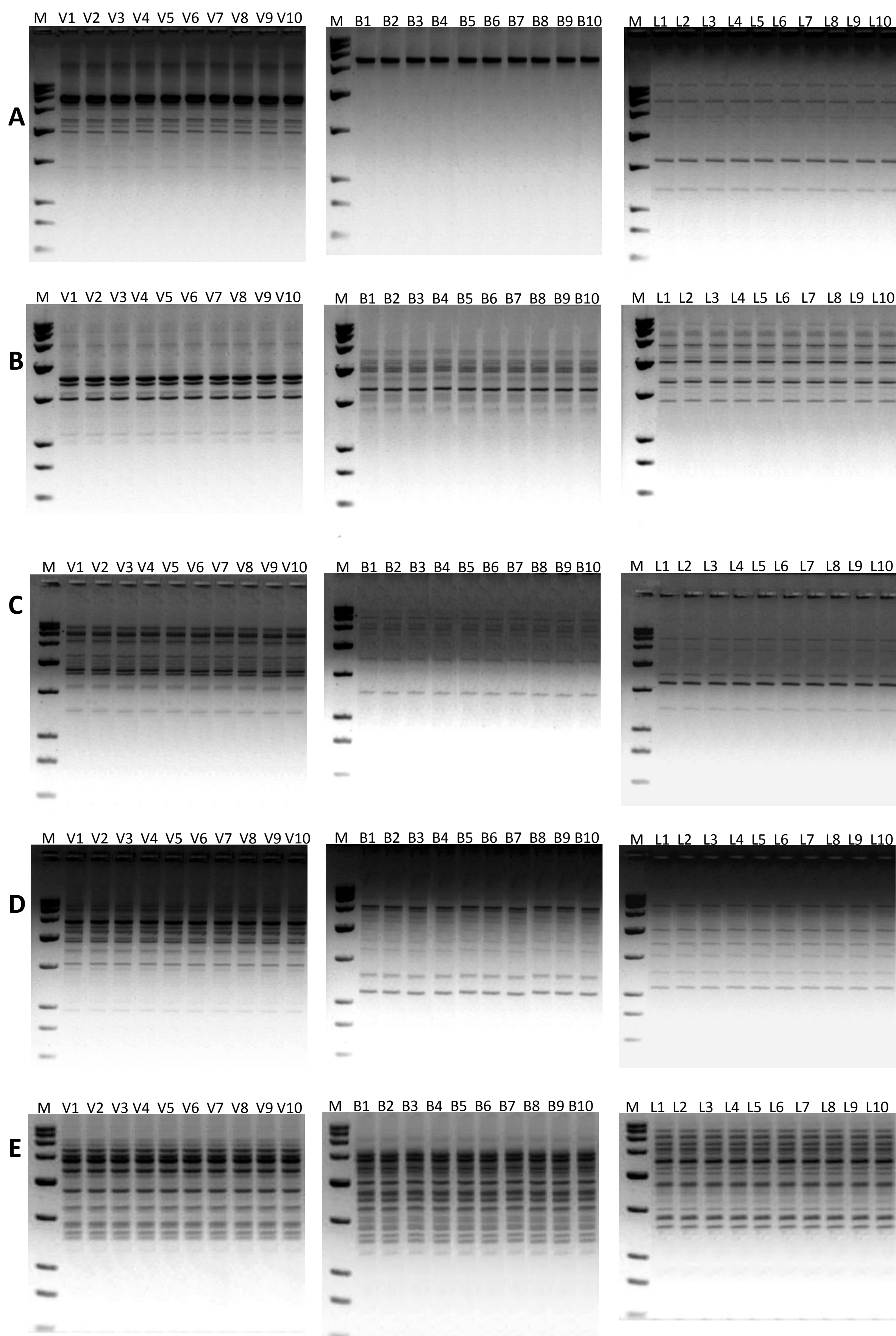

Figure 3.11 RAPD profiles generated by different primers obtained from 10 samples each of 3 commercially available banana cultivars of Mizoram. M = 100 bp marker; V1-V10 = 10 samples of Vaibalhla, B1-B10 =10 samples of Banria, L1-L10 = 10 samples of Lawngbalhla; **A** = UBC-416, **B** = UBC-418, **C** = UBC-419, **D** = UBC-420, **E** = UBC-421.

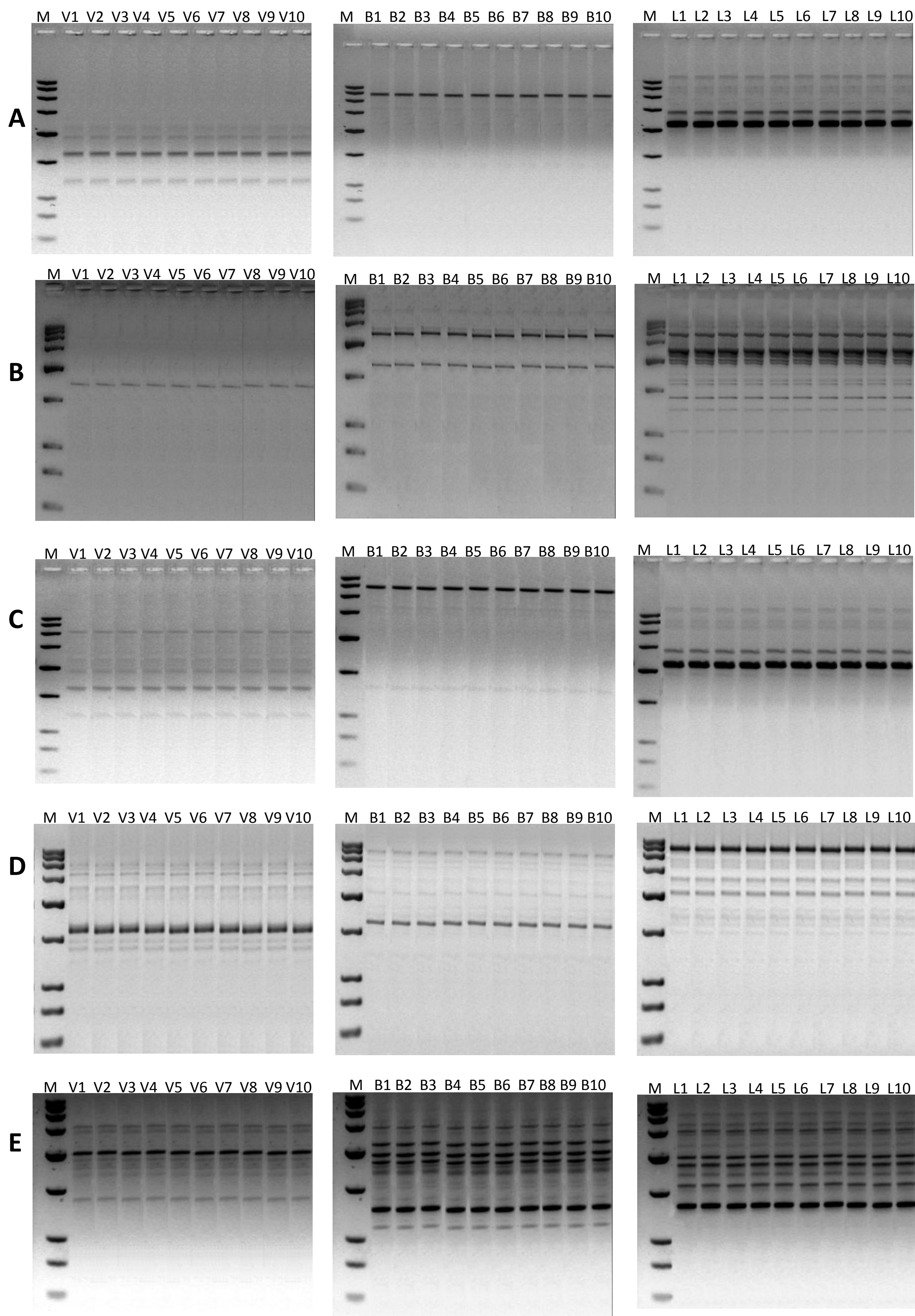

Figure 3.12 RAPD profiles generated by different primers obtained from 10 samples each of 3 commercially available banana cultivars of Mizoram. M = 100 bp marker; V1-V10 = 10 samples of Vaibalhla, B1-B10 =10 samples of Banria, L1-L10 =10 samples of Lawngbalhla; **A** = UBC-433, **B** = UBC-434, **C** = UBC-440, **D** = UBC-449, **E** = UBC-450.

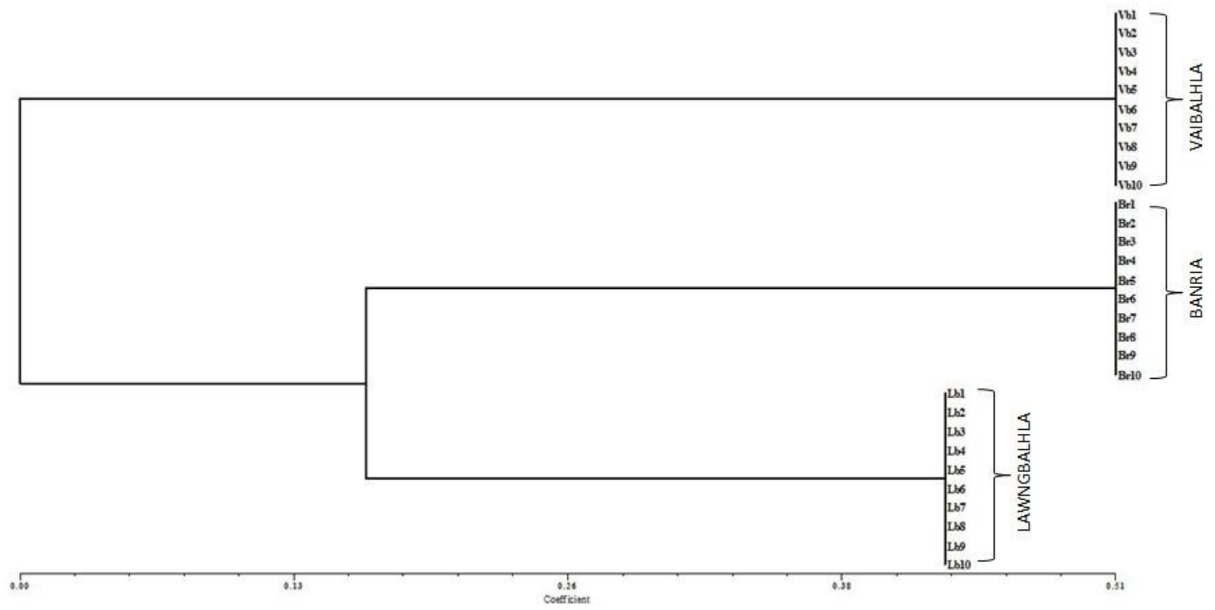

Figure 3.13 Dendrogram generated from the RAPD profiles of 10 individual plants representing three commercially available banana cultivars in Mizoram collected from Khumtung, Serchhip district.

Chapter 4

In vitro regeneration and synthetic seed production of *Musa acuminata* (AAA) cv. Vaibalhla derived from immature male flowers

CHAPTER 4

IN VITRO REGENERATION AND SYNTHETIC SEED PRODUCTION OF *MUSA ACUMINATA* (AAA) CV. VAIBALHLA DERIVED FROM IMMATURE MALE FLOWERS

4.1 Introduction

The triploid *M. acuminata* cv. Vaibalhla (AAA) is the most widely cultivated local banana in Mizoram having a characteristic sweet taste (Lalrinfela and Thangjam, 2012). However, the large-scale production of this cultivar is hampered by the lack of disease free quality planting materials. Tissue culture techniques have been used for faster and reliable means of production of large number of genetically uniform clonal planting materials. Though there are many available reports on *in vitro* propagation in banana, the standardization of specific protocols for an economically important cultivar like Vaibalhla is essential. Micropropagation using banana male floral meristems showed no detectable somaclonal variation and is found to show less risk for possible virus contamination (Harirah and Khalid, 2006) as compared to soil grown suckers. Moreover, there is an opportunity to select male buds with desirable characteristics such as greater number of hands and fruits per bunch (Resmi and Nair, 2007), which help to increase the efficiency of micropropagation and produce plantlets from the parts which could be lost during harvesting, when in normal cases, male buds serve only as food or fodder. Thus, the demand for a large number of suckers of elite cultivars can be met using inflorescence tip cultures (Mahadev *et al*., 2011).

4.2 Materials and Methods

4.2.1 Plant material

Immature male flowers obtained from the male bud of *M. acuminata* cv. Vaibalhla (AAA) maintained at the field gene bank in the Department of Biotechnology, Mizoram University, Aizawl was used as the explant source.

4.2.2 Optimization of sterilization procedure

The male buds were shortened to 4 - 6 cm in length by removing the enveloping bracts. The shortened buds were washed with labolene (Himedia Pvt. Ltd., Mumbai) for 30 minutes and rinsed thoroughly with tap water. The buds were brought under laminar air flow chamber (LAF) and then surface sterilized with 70% ethanol for 5 minutes followed by treatment with different concentrations (0, 0.5, 1, 1.5 and 2%) of sodium hypochlorite (NaOCl) for 5 minutes and finally rinsed 5 times with sterile distilled water. The sterilized buds were dissected and individual male flowers (10-20 mm) were cultured on MS basal media supplemented with BAP (2 mg/L) and NAA (0.5 mg/L). Data on the percentage of contaminated explants, number of explants dying due to NaOCl treatment and percentage of surviving explants were recorded on weekly basis.

4.2.3 Standardization of media and plant growth regulators

For the standardization of media composition individual male flowers were cultured on MS basal medium (Murashige and Skoog, 1962) supplemented with different concentrations (0, 1, 2, 3, 4 and 5 mg/L) of BAP, Kinetin, NAA or 2,4-D in single or in combinations for initial culture as well as for shoot proliferation and multiplication.

Sucrose (30%) and myo-inositol (100 mg/L) were added in all media and the pH was adjusted to 5.5 – 5.8. The media was solidified with 0.8% (w/v) agar and sterilized for 15 min at 121°C (15 lb psi pressure). Cultures were incubated at 25±1°C under 16:8 h light and dark photoperiod with light intensity of 55 $\mu M/m^2/s$ using cool white fluorescent light (Bajaj Ltd., India) and 50-60% relative humidity (RH).

4.2.4 Control of phenolic exudation

For the control of browny phenolic exudation observed in the cultures, different concentrations of ascorbic acid (0, 25, 50, 75 and 100 mg/L) was added to the culture media to evaluate their effect.

4.2.5 Plantlet regeneration

The whitish bud-like structures (WBLS) formed induced from the explants were subsequently transferred on a shoot proliferation medium containing MS media supplemented with different concentrations of BAP (2 and 4 mg/L), Kinetin (2 and 5 mg/L) and NAA (0.5 mg/L) either in single or in combinations. The number of shoots per bud and the plantlets with fully expanded leaves obtained in shoot proliferation medium were recorded. The rooting percentage and numbers per plantlet were recorded by sub-culturing in MS media with and without PGRs. Plantlets having fully expanded leaves and well-developed roots were transplanted for primary hardening in the laboratory containing sterilized soil: sand (1:1) and covered with polythene to maintain humidity. They were successfully transferred to greenhouse in pots containing farm yard manure: sand (1:1).

4.2.6 Data collection and statistical analysis

All experiments had three replicates per treatment with each replicate consisting of a petri plate with 5 explants, in a total of 15 explants per treatment. The experiments were repeated at least twice. The percentage data represented in the tables were arcsine transformed before being analyzed for significance using ANOVA (analysis of variance, $P < 0.05$). Further, the differences in means were contrasted using Duncan's new multiple range test following ANOVA. All statistical analysis was carried out using SPSS statistical software package version 16.0 (SPSS, 2007).

4.2.7 Assessment of genetic fidelity

The genomic DNA from tender leaves (100 mg) of the 8 randomly selected regenerated banana plantlets as well as from the mother plant maintained in the field was isolated by modified CTAB method (Thangjam *et al*., 2003). The quantity of the extracted DNA was estimated using Biophotometer plus (Eppendorf, Germany) by taking their absorbance at 260 nm and 280nm. RAPD-PCR amplifications were carried out with 40 RAPD primers (UBC set #5) performed in a Thermal Cycler (Bio Rad, C1000™) with the following conditions: 3 min at 94°C; 35 cycles of: 1 min at 94°C, 1 min at 36°C, 2 min at 72°C and a final extension step 10 min at 72°C. PCR reactions were carried out in a 20 µl reaction mixture volume containing 50 ng of template DNA, 100 µM dNTP mix (Himedia, India), 0.6 µM of random primers, 1× Taq DNA polymerase buffer containing 15mM $MgCl_2$ (Sigma-Aldrich Pvt. Ltd, Bangalore) and 1.0 U of Taq polymerase (Sigma-Aldrich Pvt. Ltd, Bangalore). The amplified products were resolved by electrophoresis on a 1.2% (w/v) agarose gel run in 1× TBE buffer and detected by ethidium bromide staining. The resulting fragments were scored under UV light using a gel documentation system (Bio-rad, Australia)

and analysed with Quantity one-1D software (Bio-rad, Australia). A double-digested (EcoRI and HindIII) 1 Kb λ DNA ladder (Fermentas, USA) was used as molecular marker. All the PCR reactions were repeated at least twice to check the reproducibility. For the ISSR analysis 30 primers (UBC set #9) were tested using similar amplification protocol with variable annealing temperatures and resolved on a 1.5% (w/v) agarose gel. Only the distinct, reproducible, and well-resolved fragments were scored as present (+) or absent (-).

4.2.8 Preparation of synthetic seeds

In vitro established explants which formed buds (WBLS) within 5-7 weeks of culture in MS medium supplemented with BAP (2 mg/L) and NAA (0.5 mg/L) were used for the encapsulation experiments. For the evaluation of the optimum bead forming solution, the buds were mixed with a gel of different percentage of sodium-alginate (2, 3 and 4%) prepared in MS medium. Encapsulated explants were mixed well in the alginate matrix. Using a sterile wide bore (2 mm) pipette, individual buds along with sodium-alginate is sucked and dropped one by one distant from each other in a beaker containing different concentrations of calcium chloride solution (180 and 250 mM) and kept for 20 minutes with occasional agitation. The resulting beads composed of calcium alginate enclosing buds were then tested for germination by inoculating on MS medium supplemented with different concentrations of sucrose (15 and 30 g/L) and myo-inositol (0.1 and 0.3 g/L) combinations.

4.3 Results

4.3.1 Optimization of sterilization procedure and phenolic exudation control

Prior to inoculation, 10 male flower explants each were sterilized with five different concentrations of NaOCl (0, 0.5, 1, 1.5 and 2%) for 5 minutes. Among the various treatments tested, the effective results in controlling contamination were obtained within 5 days of culture (Table 4.1). The least percentage of explant contamination (16.66) was observed with 2% NaOCl. However the explant survival was less for this concentration. The highest explant survival was observed in explants treated with 1% NaOCl with 66.66 % survival (Fig. 4.1). Thus this concentration was chosen as the optimum and used in the sterilization of the explants.

For controlling the phenolic exudations, a batch of 33 explants each were cultured in MS medium supplemented with different concentrations of ascorbic acid (0, 25, 50, 75 and 100 mg/L). Within a period of 4 weeks the levels of exudation was observed (Table 4.2). Out of the five different concentrations tested, MS medium supplemented with 75 and 100 mg/L ascorbic acid could completely (100%) reduce the unwanted brownish exudations throughout the culture period (Fig. 4.2).

4.3.2 *In vitro* regeneration

The data on the effect of MS basal medium supplemented with different concentrations and combinations of plant growth regulators (PGRs) viz. BAP, Kinetin and NAA on immature male flower explants is given in Table 4.3. All explants showed varied morphogenetic response within the first 2 weeks of culture. The first morphogenetic change observed was in the colour of the white immature male flowers turning to green followed by bulging at the base. Induction of white bud-like

structures (WBLS) was observed in the cultures by the 3rd week (Fig. 4.3) with a maximum number (4.30) of WBLS per explant in MS media supplemented with the combination of BAP (2 mg/L) and NAA (0.5 mg/L). The frequency and time taken for WBLS formation varied in the different combinations of MS media supplemented with PGRs. The induced buds were subsequently sub-cultured for shoot regeneration in MS media with combination of different PGRs resulting in the formation of shoots within 2 weeks (Fig. 4.4). The highest percentage (77.77) and number (3.51) of shoot formation was observed in MS media supplemented with a combination of Kinetin (2 mg/L) and NAA (0.5 mg/L) (Table 4.4).

The regenerated individual shoots were separated and then sub-cultured in a fresh media for further elongation. MS medium supplemented with a combination of BAP (2 mg/L) and NAA (0.5 mg/L) showed the maximum shoot length (14.44 cm). The appearance of leaves was observed from all the shoots by the 3rd week accompanied with root induction. For the evaluation of rooting efficiency, the induced roots were cut off and the shoots were transferred to fresh media containing different PGRs. Within 1 week, fresh roots appeared in varied numbers. The maximum number (7.19) and length (20.23 cm) of roots was observed in the MS basal medium without any PGRs (Fig. 4.5 H) followed by Kinetin (5 mg/L) and the least with the combination of Kinetin (2 mg/L) and NAA (0.5 mg/L) (Table 4.5).

The fully rooted plantlets were taken out from the culture bottles, washed thoroughly to remove any remains of medium, and planted in small plastic pots containing sterilized soil: sand (1:1). Plants were covered with transparent polyethylene bags to maintain adequate moisture for a week and transferred to the greenhouse (28°C day, 20°C night, 16 h day-length, and 70% humidity). After a week, the plastic covering

was removed and the plantlets were maintained in the greenhouse in plastic pots containing farm yard manure: sand (1:1) until they were transplanted to the nursery. About 96% of the hardened plants survived in the nursery. The schematic representation of the regeneration process is given in Figure 4.5.

4.3.3 Analysis of genetic fidelity of the *in vitro* raised plantlets

For the evaluation of the clonal fidelity, genomic DNA from 8 randomly selected regenerated plantlets and the mother plant were studied using RAPD and ISSR markers. A total of 40 RAPD primers were tested and 8 of them produced 20 distinct and reproducible amplicons. These 8 primers were used for the experiment. The details of the primers used and the banding patterns are given in Table 4.6. The number of bands with each primer varied from 1 (UBC-418 and UBC-449) to 4 (UBC-419 and UBC-438) with an average of 2.5 bands per primer. The representative profiles of the 8 *in vitro* derived plants and the control donor mother plant with RAPD primer (UBC-419) is shown in Figure 4.6A. All the bands were found to be monomorphic in the regenerated and the mother plant. On the other hand, after an initial screening of 30 ISSR primers, 8 of them produced 35 clear and countable amplification products ranging between 100 and 4500 bp length with no polymorphism. The optimum annealing temperature varied between 47.0 and 47.5°C according to different ISSR primers (Table 4.7). Number of scoreable bands for each primer varied from 3 (UBC-845) to 6 (UBC-834) with an average of 4.37 bands per primer. The representative profiles of the 8 *in vitro* derived plants and the control donor mother plant with ISSR primer (UBC-827) is shown in Figure 4.6B. Overall the number of bands per primer was greater in ISSR (4.37) than RAPD (2.5).

4.3.4 Encapsulation of buds

For the encapsulation of the induced buds obtained from *M. acuminata* cv. Vaibalhla (AAA), the combination of 3% sodium-alginate and 180 mM calcium chloride with the MS media was found to provide the best parameters for a good synthetic seed (Fig. 4.7). Isodiametric, compact and translucent encapsulated buds were obtained (Table 4.8). The germination potential of encapsulated buds placed on MS medium showed variable response with the initial response of browning due to the excessive phenolic secretion. Table 4.9 shows the order of successive response in terms of the potential of germination. The best reaction culture was observed in the synthetic seeds cultured on full MS medium + sucrose (30 g/L) + agar (8 g/L) + myo-inositol (0.1 g/L) with a mean germination of 6.33 out of the 7 seeds inoculated (Fig. 4.8). In other combinations the encapsulated beads deteriorate and die within 2 months of culture.

4.4 Discussion

In the present study, the successful *in vitro* regeneration of *M. acuminata* cv. Vaibalhla (AAA) plantlets were achieved using immature male flower explants. Though the use of male flowers of banana is common for developing embryogenic suspension cultures or callus (Sidha *et al*., 2007; Pérez-Hernández and Rosell-García, 2008; Sultan *et al*., 2011) limited reports have been found in the case of direct regeneration from immature male flowers (Resmi and Nair, 2007; Darvari *et al*., 2010; Mahadev *et al*., 2011).

The white bud-like structures observed in the present investigation was similar to the white buds observed from *in vitro* regenerated male flowers in different banana cultivars such as Sannachenkadali (AA), Red banana (AAA) (Resmi and Nair, 2007)

and Virupakshi (AAB) and Sirumalai (AAB) (Mahadev *et al*., 2011). Buds primarily appeared within 2 months in earlier reports (Darvari *et al*., 2010; Mahadev *et al*., 2011) when cultured in high BAP concentrations (8 mg/L, 5 mg/L) while the present study shows the formation of WBLS only within 5-7 weeks in MS medium supplemented with a lower concentration of BAP (2 mg/L) and NAA (0.5 mg/L) in combination. When the concentration of BAP was increased (4 mg/L and 5 mg/L), the time taken to form WBLS was lesser (3-5 weeks), but the total number of WBLS formation decreased and thus it was less desirable. Similar responses were observed from cultivars Berangan (AAA), Rastali (AAB), Nangka (AAB) and Abu (ABB) (Darvari *et al*., 2010).

The highest percentage and number of shoot formation observed in MS media supplemented with a combination of Kinetin (2 mg/L) and NAA (0.5 mg/L) shows that BAP does not have a significant role in shoot formation, where MS media supplemented with BAP produced lesser number of shoots. These responses clearly indicate that though both BAP and Kinetin are cytokinins (purine derivatives) causing similar effects in plants, their efforts on shoot proliferation vis-à-vis multiplication are different for cv. Vaibalhla (AAA). A similar kind of response was also observed in cv. Nanjanagudu Rasabale (AAB) (Venkatachalam *et al*., 2007).

Rooting was maximum in MS basal media without any PGR supplementation which shows that addition of auxin or cytokinins did not promote any significant increase in the rooting frequency. This is in agreement with the earlier observations in AAB cvs. Virupakshi and Sirumalai (Mahadev *et al*., 2011).

The major principle involved in the alginate encapsulation process is that the sodium alginate droplets containing the plant material to be encapsulated, when dropped into

the calcium chloride solution form round and firm beads due to ion exchange between the Na^{+} in sodium alginate with Ca^{2+} in the calcium chloride solution (Saiprasad, 2001). The hardness or rigidity of the capsule mainly depends upon the number of sodium ions exchanged with calcium ions. In the present study, 3% sodium-alginate and 180 mM calcium chloride was found to be optimum in giving the best encapsulation for the buds resulting in a isodiametric, compact and translucent beads. The use of full strength MS medium yielded a very high plant conversion response (90.42%) suggesting that ingredients in the encapsulation mixture contribute to the developing encapsulated buds. A synthetic endosperm is known to contain nutrients including carbon sources for optimum germination and plantlet conversion (Antonietta *et al*., 1999). The usefulness of full strength MS medium for high conversion frequency was also reported in *Solanum melongena* Rao and Singh (1991) and sandalwood (Bapat and Rao, 1988). However, in papaya, half-strength MS medium was found to be effective for the germination of encapsulated somatic embryos (Castillo *et al*., 1998). For the germination of synthetic seeds developed from banana cv. Rasthali, the use of full strength MS medium yielded the highest plant conversion response (66%) and shoot and root growth compared to other treatments (Ganapathi *et al*., 2001a).

True-to-true type clonal fidelity is one of the most important prerequisites in the micropropagation of any crop species. A major problem often encountered with the *in vitro* cultures is the presence of somaclonal variation amongst subclones of one parental line, arising as a direct consequence of *in vitro* culture of plant cells, tissues, or organs. In banana micropropagation, though the protocol is known to yield clonal material, there are reports of somaclonal variation especially in cv. Williams

(Damasco *et al*., 1996). A good analysis of genetic stability of plantlets can be made by using a combination of two types of markers that amplify different regions of the genome (Lattoo *et al*., 2006; Ray *et al*., 2006; Faisal *et al*., 2012; Phulwaria *et al*., 2013). Hence, in the present study, two PCR-based techniques, RAPD and ISSR, were adopted for evaluation of clonal fidelity in banana plantlets.

While genetic variations are exceptions, most of the organized cultures, especially the shoot tips maintain strict genotypic and phenotypic stability under tissue culture conditions (Bennici *et al*., 2004). This is also applicable to cv. Vaibalhla (AAA) since no variations were observed using the set of primers tested with the randomly selected regenerated plantlets. It has been found that high level of growth regulators caused variants like suppression of shoot length leading to morphological abnormalities and genetic changes in other genotypes of banana (Shenoy and Vasil, 1992; Martin *et al*., 2006). And thus in the present study, low levels of growth regulators were used.

The number of bands per primer was greater in ISSR (4.37) than RAPD (2.5).The reason for this could be because of high melting temperature for ISSR primers, which allows much more rigorous annealing conditions and consequently, more precise and repeatable amplification. It has been proven that ISSR fingerprints detect more polymorphic loci when compared with RAPD fingerprinting (Devarumath *et al*., 2002; Bhatia *et al*., 2011).

Table 4.1 Effect of sodium hypochlorite (NaOCl) treatment (5 min) for surface sterilization of male inflorescence explants of *M. acuminata* cv. Vaibalhla (AAA) after 1 week of culture.

NaOCl (%)	Total no. of explants	Percent of contamination (%) (Mean ± SE)	No. of explants dying due to NaOCl treatment (Mean±SE)	Survival % (Mean±SE)
0.0	10	93.33 ± 6.14a	0.00 ± 0.00	6.66 ± 6.14a
0.5	10	80.00 ± 4.27b	0.66 ± 0.33	13.33 ± 2.71a
1.0	10	23.33 ± 2.07cf	1.00 ± 0.00	66.66 ± 2.00b
1.5	10	23.33 ± 2.21df	4.00 ± 0.57	36.66 ± 2.00ce
2.0	10	16.66 ± 2.71ef	5.66 ± 0.33	25.00 ± 2.21de

Medium: MS + PGRs. Mean (±) followed by the same letter(s) in each column are not significantly different at $P<0.05$ using Duncan's new multiple range test.

Table 4.2 Effect of ascorbic acid in controlling the phenolic exudation given from male inflorescence explants of *M. acuminata* cv. Vaibalhla (AAA) after 4 weeks of culture.

Ascorbic acid (mg/L)	Total no. of explants	Explants without exudation (%) (Mean ± SE)
0.0	33	0.00 ± 0.00a
25.0	33	39.39 ± 1.76b
50.0	33	66.66 ± 0.00c
75.0	33	100.00 ± 0.00df
100.0	33	100.00 ± 0.00ef

Medium: MS + PGRs. Mean (±) followed by the same letter(s) in each column are not significantly different at $P<0.05$ using Duncan's new multiple range test

Table 4.3 Effect of plant growth regulators on immature male flower explants of *M. acuminata* cv. Vaibalhla (AAA) for WBLS formation.

PGR (mg/L)			Time taken to form WBLS (weeks)	Percentage of explants forming WBLS (%) (Mean ± SE)	Number of WBLS per explant (Mean ± SE)
BAP	Kin	NAA			
2.0	0.0	0.0	10-12	33.33 ± 2.35a	1.40 ± 0.10a
3.0	0.0	0.0	7-10	8.88 ± 2.15bk	1.16 ± 0.16a
4.0	0.0	0.0	3-5	17.77 ± 1.71cl	2.05 ± 0.24bj
5.0	0.0	0.0	3-5	31.10 ± 1.39a	1.78 ± 0.11aj
0.0	1.0	0.0	20-22	15.55 ± 1.71dkl	2.16 ± 0.16cj
0.0	2.0	0.0	7-9	24.44 ± 1.50al	1.66 ± 0.08aj
0.0	3.0	0.0	7-9	8.88 ± 2.15ek	2.33± 0.33dj
0.0	5.0	0.0	7-9	73.33 ± 2.51fmn	2.06 ± 0.16ej
2.0	0.0	0.5	5-7	71.10 ± 1.39gmn	4.30 ± 0.10f
4.0	0.0	0.5	5-7	35.55 ± 2.71a	2.49 ± 0.09gj
0.0	2.0	0.5	10-12	55.55 ± 1.28ho	1.63 ± 0.14aj
2.0	2.0	0.0	8-10	53.32 ± 3.88io	2.12 ± 0.01hj
3.0	3.0	0.0	5-7	62.22 ± 1.32jno	2.10 ± 0.11ij

Medium: MS + PGRs. Mean (±) followed by the same letter(s) in each column are not significantly different at $P<0.05$ using Duncan's new multiple range test.

Table 4.4 Effect of plant growth regulators on shoot formation from the buds derived from immature male flower explants of *M. acuminata* cv. Vaibalhla (AAA) after 16 weeks of culture.

PGR (mg/L)			WBLS forming shoots (%)	No. of shoots per explant (Mean ± SE)	Length of shoots(cm) (Mean ± SE)
BAP	Kin	NAA			
0.0	5.0	0.0	46.66 ± 2.21a	1.69 ± 0.10a	8.56 ± 0.41a
2.0	0.0	0.5	48.88 ± 1.27a	2.31 ± 0.08b	14.44 ± 0.15b
4.0	0.0	0.5	31.10 ± 1.39b	1.21 ± 0.01c	12.97 ± 0.06c
0.0	2.0	0.5	77.77 ± 1.51c	3.51 ± 0.03d	10.50 ± 0.06d

Medium: MS + PGRs. Mean (±) followed by the same letter(s) in each column are not significantly different at P<0.05 using Duncan's new multiple range test.

Table 4.5 Effect of plant growth regulators on root formation from shoots derived from immature male flower explants of *M. acuminata* cv. Vaibalhla (AAA).

PGR (mg/L)			Length of roots (cm) (Mean ± SE)	No. of roots per plantlet (Mean ± SE)
BAP	Kin	NAA		
0.0	0.0	0.0	20.23 ± 0.04a	7.19 ± 0.07a
0.0	5.0	0.0	17.85 ± 0.28bf	5.28 ± 0.12b
2.0	0.0	0.5	19.65 ± 0.18c	4.64 ± 0.08cf
4.0	0.0	0.5	17.30 ± 0.06d	4.39 ± 0.13df
0.0	2.0	0.5	18.18 ± 0.13ef	4.33 ± 0.07ef

Medium: MS + PGRs. Mean (±) followed by the same letter(s) in each column are not significantly different at P<0.05 using Duncan's new multiple range test.

Table 4.6 Details of the RAPD profile obtained from genomic DNA isolated from *in vitro* regenerated plantlets of *M. acuminata* cv. Vaibalhla (AAA).

RAPD Primer	Primer Sequence (5'-3')	Tm (°C)	No. of monomorphic bands	No. of polymorphic bands	Total no. of bands amplified	Size range (bp)
UBC-418	GAGGAAGCTT	36°C	1	0	1	800-1500
UBC-419	TACGTGCCCG	36°C	4	0	4	500-3500
UBC-420	GCAGGGTTCG	36°C	2	0	2	800-3000
UBC-421	ACGGCCCACC	36°C	2	0	2	800-3000
UBC-433	TCACGTGCCT	36°C	3	0	3	100-3500
UBC-438	AGACGGCCG	36°C	4	0	4	800-3000
UBC-449	GAGGTTCAAC	36°C	1	0	1	100-800
UBC-450	CGGAGAGCCA	36°C	3	0	3	100-2000
	Total		20	0	20	

Table 4.7 Details of the ISSR profile obtained from genomic DNA isolated from *in vitro* regenerated plantlets of *M. acuminata* cv. Vaibalhla (AAA).

ISSR Primer	Primer Sequence (5'-3')	Tm (°C)	No. of monomorphic bands	No. of polymorphic bands	Total no. of bands amplified	Size range (bp)
UBC-827	$(AC)_8G$	47.5°C	4	0	4	800-3500
UBC-829	$(TG)_8C$	47°C	4	0	4	800-2000
UBC-830	$(TG)_8G$	47°C	4	0	4	500-2000
UBC-834	$(AG)_8YT$	47°C	6	0	6	100-2000
UBC-840	$(GA)_8YT$	47°C	5	0	5	100-1500
UBC-843	$(CT)_8RA$	47°C	4	0	4	100-4500
UBC-844	$(CT)_8RC$	47.5°C	5	0	5	800-3000
UBC-845	$(CT)_8RG$	47°C	3	0	3	800-3500
	Total		35	0	35	

Where Y = C, T; R = A, G

Table 4.8 Effect of sodium-alginate and calcium chloride on type and colour of encapsulation on buds of *M. acuminata* cv. Vaibalhla (AAA).

Sodium alginate (%)	Calcium Chloride (mM)	Type of encapsulation	Colour of encapsulation
2.0	180.0	Loose	Transparent
2.0	250.0	Fragile and soft	Transparent
3.0	180.0	Isodiametric and compact	Transparent
3.0	250.0	Isodiametric and hard	Transparent
4.0	180.0	Isodiametric and hard	Transparent
4.0	250.0	Isodiametric and hard	Whitish

Table 4.9 Effect of different MS media composition on the germination of encapsulated seeds obtained from bud derived male flower of *M. acuminata* cv. Vaibalhla (AAA).

Chemicals used (g/L)		No. of artificial seeds inoculated	No. of seeds showing germination (Mean ± SE)
Sucrose	Myo-inositol		
0.0	0.0	7	0.00 ± 0.00a
15.0	0.1	7	2.66 ± 0.33b
30.0	0.1	7	6.33 ± 0.33c
30.0	0.3	7	4.33 ± 0.33d

Medium: MS + PGRs. Mean (±) followed by the same letter(s) in each column are not significantly different at P<0.05 using Duncan’s new multiple range test.

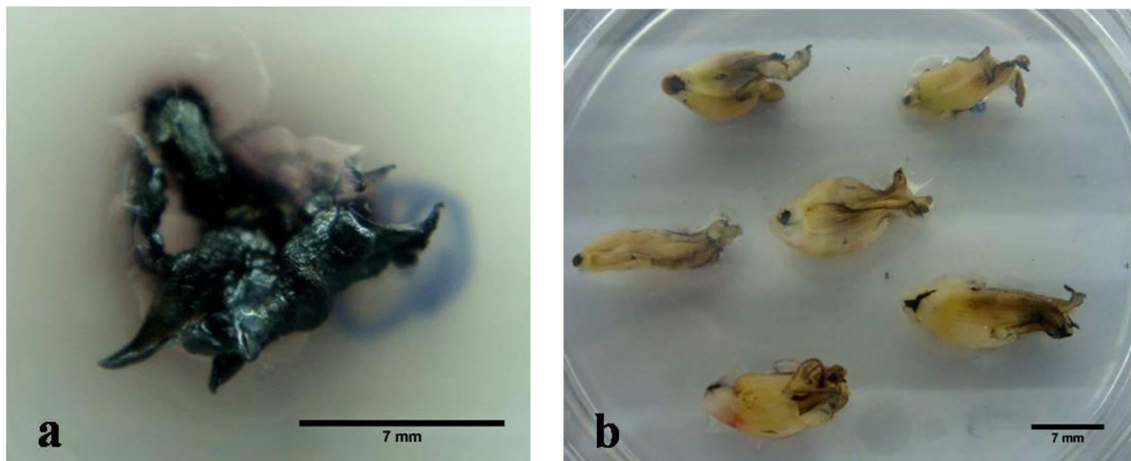

Figure 4.1 Standardization of sterilization of immature male flowers of *M. acuminata* cv. Vaibalhla (AAA) for aseptic culture**: a**- Male flower with 0% NaOCl treatment showing bacterial contamination after 5 days; **b**- Male flowers treated with 1% NaOCl showing no sign of contamination after 5 days.

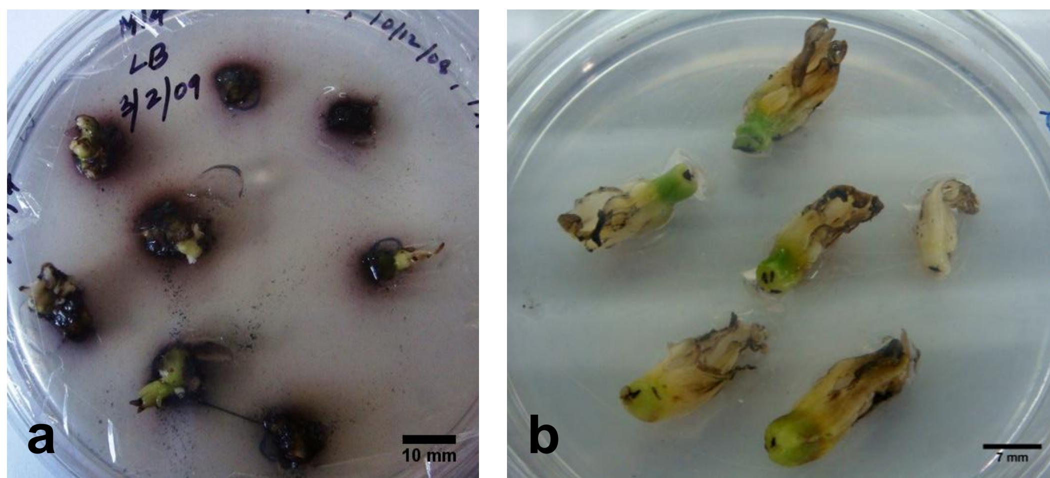

Figure 4.2 Control of phenolic exudation on the immature male flower cultures of *M. acuminata* cv. Vaibalhla (AAA): **a** – Phenolic exudation seen in 4 weeks cultured male flower explants without ascorbic acid treatment; **b** – 4 weeks cultured male flower explants free from phenolic exudation after treatment with 75 mg/L ascorbic acid.

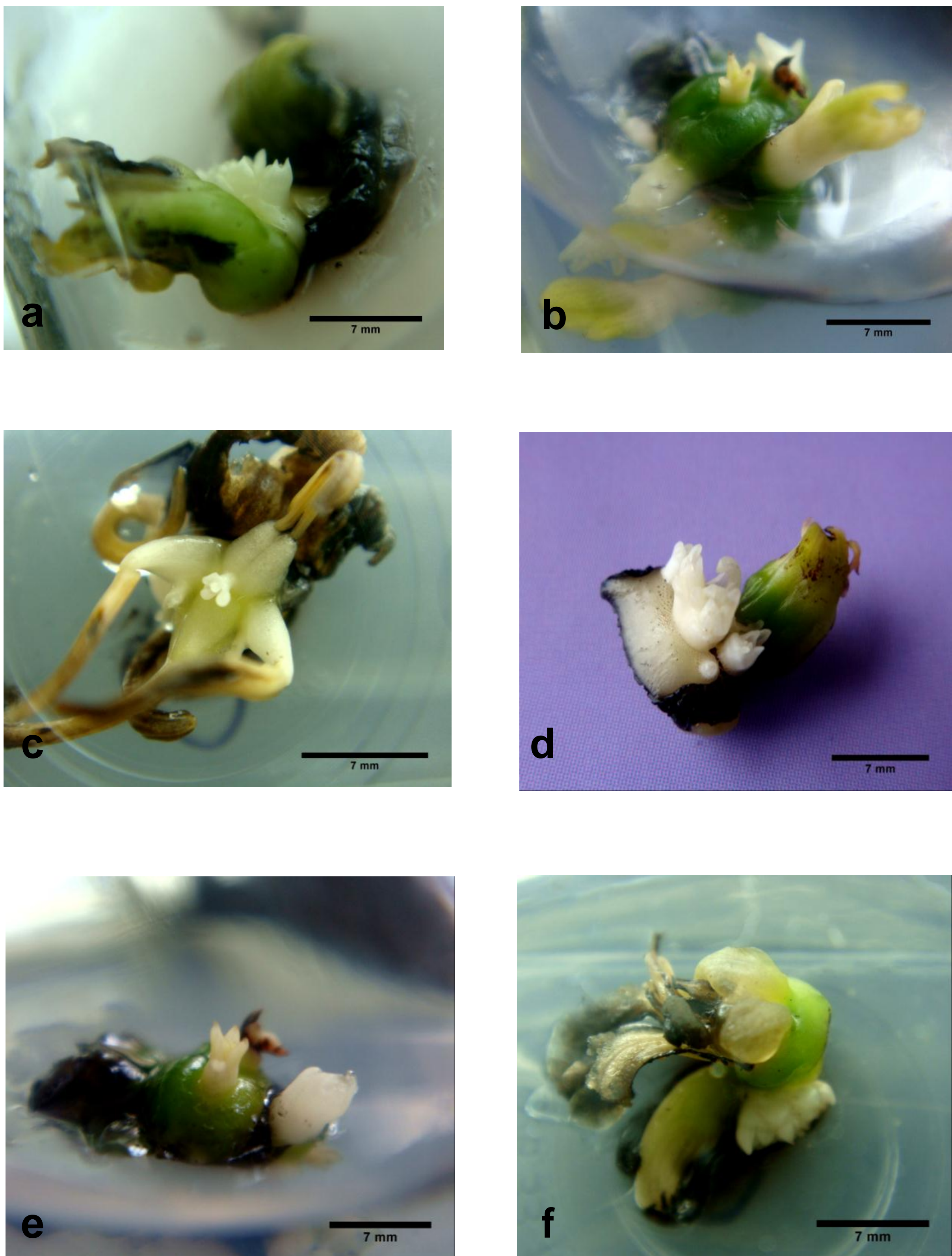

Figure 4.3 White bud-like structures (WBLS) observed from 5-10 weeks cultured male flowers of *M. acuminata* cv. Vaibalhla (AAA). **a** – Formation of WBLS in MS + Kn (2 mg/L) + NAA (0.5 mg/L) after 10 weeks; **b** – Formation of WBLS in MS + BAP (2 mg/L) + NAA (0.5 mg/L) after 5 weeks; **c** – Formation of WBLS in MS + Kn (5 mg/L) after 7 weeks; **d** – Formation of WBLS in MS + BAP (3 mg/L) + Kn (3 mg/L) after 5 weeks; **e** – Formation of WBLS in MS + BAP (2 mg/L) + Kn (2 mg/L) after 8 weeks; **f** – Formation of WBLS in male flowers cultured in MS + BAP (4 mg/L) + NAA (0.5 mg/L) after 5 weeks.

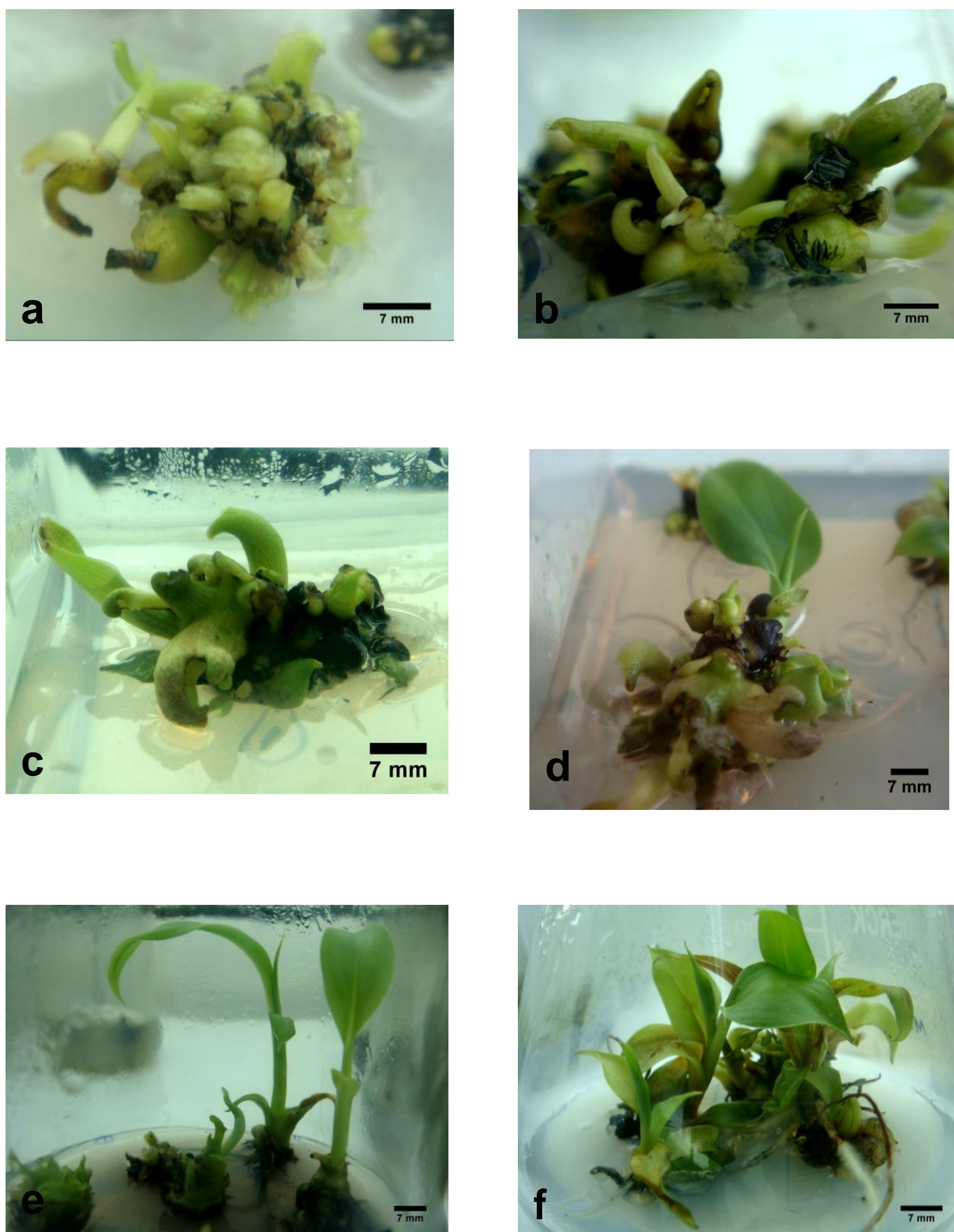

Figure 4.4 a-f – Different stages of shoot and leaf regeneration from WBLS derived from male flowers of *M. acuminata* cv. Vaibalhla (AAA) after 16 weeks from initial culture.

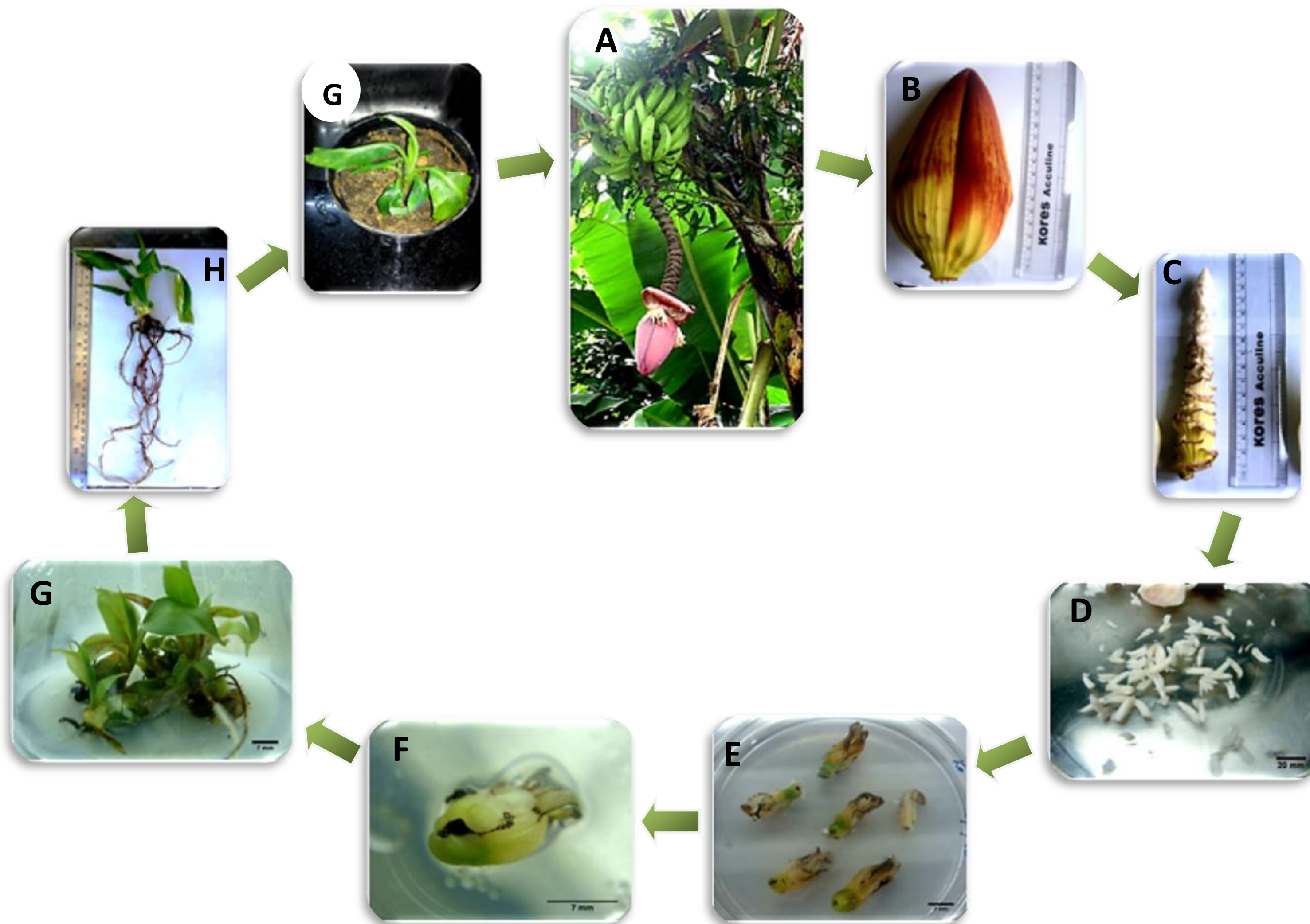

Figure 4.5 Schematic diagram of different stages of the *in vitro* regeneration of *M. acuminata* cv. Vaibalhla (AAA) derived from immature male flower explants: **A** Mother plant bearing fruits and male bud. **B** Male floral bud. **C** Reduced floral bud (4 cm.). **D** Individual immature male flowers dissected separately. **E** Explant turning green in MS+BAP (2.0mg/L) + NAA (0.5 mg/L) after 1 week. **F** Explant bulging within 2 weeks. **G** *In vitro* shoot clusters **H** *In vitro* regenerated plantlet with shoots and roots. **H** Secondary hardened plant.

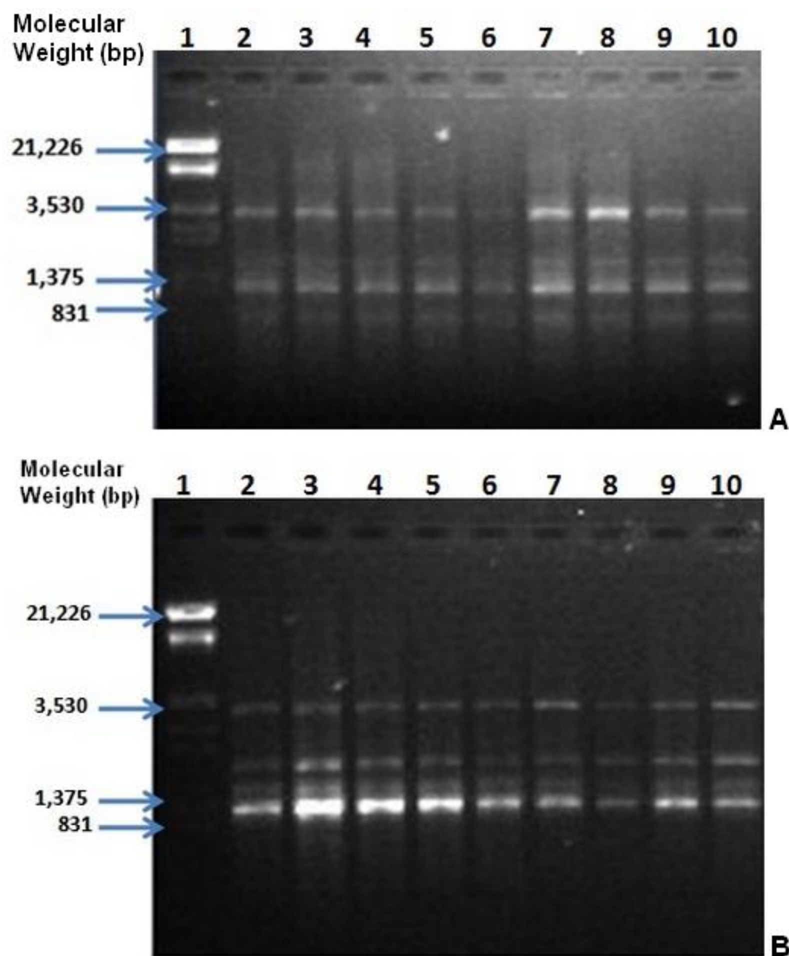

Figure 4.6 RAPD and ISSR profile of the *in vitro* regenerated plantlets derived from male floral explants of *M. acuminata* cv. Vaibalhla (AAA). **A** – Amplification with UBC RAPD Primer #419; **B** – Amplification with UBC ISSR Primer #827. Lane 1: lambda DNA HindIII marker, 2 donor plant, 3-10 regenerated plantlets

Figure 4.7 Encapsulation of buds derived from immature male flower explants of *M. acuminata* cv. Vaibalhla. Buds encapsulated with 3 % Na-alginate and 180 mM.

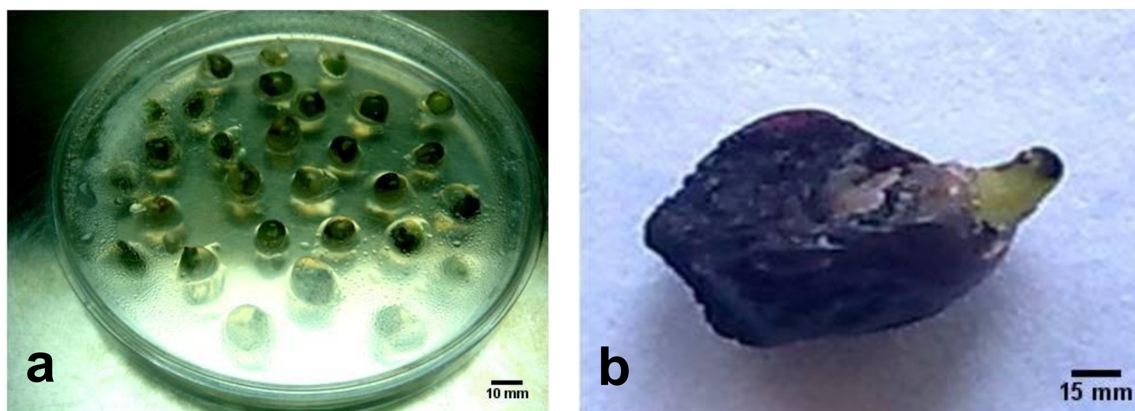

Figure 4.8 Germination of encapsulated seeds: **a** – Encapsulated seeds of banana cultured in media for germination; **b** – Germination of artificial banana seed.

Chapter 5

Studies on *in vitro* regeneration of *Musa acuminata* Colla cv. Grand Naine (AAA)

CHAPTER 5

STUDIES ON *IN VITRO* REGENERATION OF *MUSA ACUMINATA* COLLA CV. GRAND NAINE (AAA)

5.1 Introduction

Musa acuminata Colla var. Grand Naine (AAA) is the most widely dessert banana grown all over the world (Côte *et al*., 1996). It was first introduced in Mizoram during the year 2005 under the horticulture technology mission, Government of India (Exim Bank, 2009). The purpose of introduction was to increase domestic produce and to improve quality. However, diseases and pests have been hampering the fruit production. Hence, there is a need for the large-scale sustainable production of this popular variety in which large number of superior quality planting materials is required. Micropropagation of this important cultivar through tissue culture technique can offer rapid and reliable means of producing large number of genetically uniform clonal planting material within a short time.

Even though several reports have been made for *in vitro* propagation of this cultivar, the standardization of a specific protocol is essential, keeping in mind that with the differences in geographical and climatic conditions of Mizoram, there is a possibility of morphological and genetical changes after its introduction. The present study was carried out with an attempt to improve the *in vitro* regeneration protocol of *M. acuminata* Colla var. Grand Naine (AAA) by evaluating the effect of MS medium supplemented with different plant growth regulators using immature male flowers as explants and to evaluate the genetic fidelity of the regenerated plantlets using RAPD and ISSR markers.

5.2 Materials and Methods:

5.2.1 *In vitro* regeneration

Healthy looking plants of Grand Naine maintained at the Horticulture Centre, Chite, Aizawl were selected as mother plant for the study. The male buds were shortened to 4 - 6 cm in length by removing the enveloping bracts, washed with labolene for 30 minutes, and rinsed with tap water. The explants were brought under laminar air flow chamber where sterilization was proceeded with 70% (v/v) ethanol for 5 minutes, then with 1% sodium hypochlorite for 5 minutes and finally rinsed with sterile distilled water (4-5 times). The sterilized buds were dissected under aseptic conditions and individual male flowers of size 10mm – 20 mm were kept aside for culture.

Individual male flowers were transferred to MS basal medium supplemented with different concentrations of plant growth regulators (PGR) viz. 2,4-D (2 mg/L), NAA (0.5 mg/L), NAA (1 mg/L), BAP (1 mg/L) and BAP (2 mg/L) along with a control without any PGR. Sucrose (30%) and myo-inositol (100 mg/L) were added in all media and pH was adjusted to 5.5 – 5.8. They were then solidified with 0.8% (w/v) agar and sterilized for 15 min at 121°C (15 lb psi pressure). Cultures were incubated at 25±1°C under 16:8 h light and dark photoperiod with light intensity of 55 $\mu M/m^2/s$ using cool white fluorescent light (Bajaj Ltd., India) and 50-60% relative humidity (RH). All the cultures were subcultured into a fresh medium containing the same PGR every fourth week.

The number of whitish bud-like structures (WBLS) formed per explant was recorded and subsequently transferred on to the same medium. The number of shoots per bud and the plantlets with fully expanded leaves obtained in shoot proliferation medium

were recorded. The evaluation of root numbers per plantlet were recorded by sub-culturing in MS media with and without PGRs. Plantlets having fully expanded leaves and well-developed roots were transplanted for primary hardening in the laboratory containing sterilized soil: sand (1:1) and covered with polythene to maintain humidity. They were successfully transferred to greenhouse in pots containing farm yard manure: sand (1:1). The established plants were apparently uniform morphologically and did not show any detectable variation. Fresh leaves were collected for RAPD and ISSR analysis. For all experiments conducted, control explants were also cultured on MS media without any supplementation of plant growth regulator.

5.2.2 Statistical analysis

All experiments had three replicates per treatment with each replicate consisting of a petri plate with 5 explants, in a total of 15 explants per treatment. The experiments were repeated at least twice. The differences in means were contrasted using Duncan's new multiple range test following ANOVA (analysis of variance, $P < 0.05$). All statistical analysis was carried out using SPSS statistical software package version 16.0 (SPSS, 2007).

5.2.3 Assessment of genetic fidelity

The genomic DNA from tender leaves (100 mg) of the 5 randomly selected regenerated banana plantlets as well as from the mother plant maintained in the field was isolated by modified CTAB method (Thangjam *et al*., 2003) The quantity of the extracted DNA was estimated using Biophotometer plus (Eppendorf, Germany) by taking their absorbance at 260 nm and 280nm. RAPD-PCR amplifications were carried out with 40 RAPD primers (UBC set #5) performed in a Thermal Cycler (Bio

Rad, C1000™) with the following conditions: 3 min at 94°C; 35 cycles of: 1 min at 94°C, 1 min at 36°C, 2 min at 72°C and a final extension step 10 min at 72°C. PCR reactions were carried out in a 20 µl reaction mixture volume containing 50 ng of template DNA, 100 µM dNTP mix (Himedia, India), 0.6 µM of random primers, 1× Taq DNA polymerase buffer containing 15mM $MgCl_2$ (Sigma-Aldrich Pvt. Ltd, Bangalore) and 1.0 U of Taq polymerase (Sigma-Aldrich Pvt. Ltd, Bangalore). The amplified products were resolved by electrophoresis on a 1.2% (w/v) agarose gel run in 1× TBE buffer and detected by ethidium bromide staining. The resulting fragments were scored under UV light using a gel documentation system (Bio-rad, Australia) and analysed with Quantity one-1D software (Bio-rad, Australia). A double-digested (EcoRI and HindIII) 1 Kb λ DNA ladder (Fermentas, USA) was used as molecular marker. All the PCR reactions were repeated at least twice to check the reproducibility. For the ISSR analysis 30 primers (UBC set #9) were tested using similar amplification protocol with variable annealing temperatures and resolved on a 1.5% (w/v) agarose gel. Only the distinct, reproducible, and well-resolved fragments were scored as present (+) or absent (-).

5.3 Results

5.3.1 *In vitro* regeneration

In the present study, the successful *in vitro* regeneration of *M. acuminata* Colla var. Grand Naine (AAA) plantlets were achieved using immature male flower explants.

The data on the effect of MS basal medium supplemented with various PGRs viz. BAP, 2,4-D and NAA on the immature male flower explants of *M. acuminata* Colla var. Grand Naine (AAA) is given in Table 5.1. All explants showed varied

morphogenetic response within 2 weeks of culture. The first change observed was the conversion of colour of the white immature male flowers to green within 1-2 weeks, followed by bulging at the base within 5-7 weeks. WBLS were observed in MS media supplemented with BAP (1, 2 mg/L) and NAA (1 mg/L) by the end of 5 weeks (Fig. 5.1 A-D) where induction of WBLS was maximum (7.22) in MS media supplemented with BAP (2 mg/L). However, WBLS formed in media containing NAA did not show further organogenesis and died. Further formation of shoot buds, leaves and roots were observed in MS media supplemented with BAP. Out of the 5 different MS media supplementation tested, MS media supplemented with BAP (1 mg/L) and BAP (2 mg/L) were the only media which could regenerate a whole plantlet from the male flowers.

The induced buds were subsequently sub-cultured in MS media supplemented with similar PGRs from where it was initially cultured for shoot regeneration which responded with the formation of shoots within a week (Fig. 5.2 A-F). The highest number of shoot per explant (5.73) and leaves per explant (15.13) were observed in MS media supplemented with BAP (2 mg/L) (Table 5.2). Induction of roots was also observed along with shoot formation. For the evaluation of rooting efficiency in different media the induced roots were cut off and the shoots were transferred to fresh media containing BAP (1, 2 mg/L) and MS basal medium without any PGR. Within 1 week fresh roots appeared in varied numbers and the maximum number (14.73) of roots was observed in the MS basal medium without any PGR followed by BAP (2 mg/L).

Plantlets obtained from MS media supplemented with BAP (1 and 2 mg/L) were compared. Even though the number of productions of buds, multiple shoots and

leaves were found higher in MS media supplemented with BAP (2 mg/L), it has also been observed that the time taken to develop a fully regenerated plantlet from MS media supplemented with BAP (1 mg/L) was lesser since formation of roots took longer time in MS media supplemented with BAP (2 mg/L) (data not shown).

The fully rooted plantlets were taken out from the culture bottles, washed thoroughly to remove any remains of medium, and planted in small plastic pots containing sterilized soil: sand (1:1) (Fig. 5.3). Plants were covered with transparent polyethylene bags to maintain adequate moisture for a week and transferred to the greenhouse (28°C day, 20°C night, 16 h day-length, and 70% humidity). After a week, the plastic covering was removed and the plantlets were maintained in the greenhouse in plastic pots containing farm yard manure: sand (1:1) until they were transplanted to the nursery. About 98% of the hardened plants survived in the nursery.

5.3.2 Analysis of genetic fidelity of the *in vitro* raised plantlets

The RAPD and ISSR banding patterns of the 5 randomly selected *in vitro* derived plants compared with the donor mother plant confirms their genetic stability. The earlier RAPD primers used in genetic fidelity testing for the *in vitro* plantlets of *M. acuminata* cv. Vaibalhla (AAA) (Chapter 4) were also used for the present investigation in which the 8 primers produced 18 distinct and reproducible amplicons in the size range of 100–3000 bp, All bands were found to be present in mother plant as well as in all the sub-cloned plantlets in which none showed polymorphism (Table 5.3). The number of bands with each primer varied from 1 (UBC-418 and UBC-449) to 4 (UBC-438) with an average of 2.25 bands per primer. On the other hand, after an initial screening of 30 ISSR primers, 8 of them produced 33 clear and countable amplification products ranging between 100 and 4500 bp length with no

polymorphism. The optimum annealing temperature varied between 47.0 and 47.5°C according to different ISSR primers (Table 5.4). Number of scoreable bands for each primer varied from 3 (UBC-845) to 6 (UBC-834) with an average of 4.125 bands per primer. The number of bands per primer was greater in ISSR (4.125) than RAPD (2.25). The representative profiles of the 5 *in vitro* derived plants and the control donor mother plant with RAPD primer (UBC-450) and ISSR primer (UBC-843) are shown in Figure 5.4. The number of bands per primer was greater in ISSR (4.125) than RAPD (2.25).

5.4 Discussion

Though there are many reports on the *in vitro* regeneration of Grand Naine, most reports were based on developing somatic embryos (Escalant *et al*., 1994; Côte *et al*., 1996; Navarro *et al*., 1997; Sagi *et al*., 1998; Becker *et al*., 2000; Sidha *et al*., 2006; Remakanthan *et al*., 2013) with the use of a combination of different PGRs, whereas the present study reports the development of an efficient *in vitro* regeneration protocol for Grand Naine by using low concentration of a single PGR - BAP (2 mg/L). Ganapathi *et al*. 2001 reported that male flowers are the most responsive starting material for initiating embryogenic cultures of Grand Naine (Escalant *et al*., 1994; Cote *et al*., 1996; Navarro *et al*., 1997; Sagi *et al*., 1998; Becker *et al*., 2000), while the present study reports the use of male flowers for direct regeneration of Grand Naine without any polymorphism among the regenerated plantlets, where fully developed plantlets were obtained within a very short period of 20 weeks.

Table 5.1 Morphogenetic response of the immature male flower explants of *M. acuminata* Colla var. Grand Naine (AAA) to MS basal medium supplemented with various plant growth regulators (PGR).

PGR	Order of morphogenetic response in weeks					
	Browny exudation	Change of colour	Bulging	White bud-like structures (WBLS)	Shoot appearance	Leaf induction
2,4-D (2.0 mg/L)	1 (100)	2 (100)	7 (100)	-	-	-
NAA (0.5 mg/L)	1 (100)	1 (100)	5 (100)	-	-	-
NAA (1.0 mg/L)	1 (100)	1 (100)	5 (77.7)	5 (55.5)	-	-
BAP (1.0 mg/L)	1 (100)	1 (100)	6 (100)	4 (100)	5 (100)	15 (100)
BAP (2.0 mg/L)	1 (100)	1 (100)	6 (100)	4 (100)	4 (100)	13 (100)

Values in parentheses are the mean percentage of explants showing response
- absence of data

Table 5.2 Effect of plant growth regulators on the formation of WBLS, shoots, leaves and roots derived from immature male flower explants of *M. acuminata* Colla var. Grand Naine (AAA).

PGR	No. of wbls/explant (4 weeks after intial culture)	No. of shoots/explant (5 weeks after WBLS formation)	No. of leaves/explant (15 weeks after WBLS formation)	No. of roots/plantlet (1 weeks after transferring to fresh media)
Control	0.00 ± 0.00a	0.00 ± 0.00a	0.00 ± 0.00a	14.73 ± 0.040a
BAP (1.0 mg/L)	4.04 ± 0.058b	3.35 ± 0.095b	9.59 ± 0.037b	10.10 ± 0.023b
BAP (2.0 mg/L)	7.22 ± 0.057c	5.73 ± 0.040c	15.13 ± 0.040c	13.35 ± 0.078c

Medium: MS + PGRs. Mean (±) followed by the same letter(s) in each column are not significantly different at $P<0.05$ using Duncan's new multiple range test.

Table 5.3 Details of the RAPD profile obtained from genomic DNA isolated from regenerated *M. acuminata* Colla var. Grand Naine (AAA) plantlets and the mother plant.

RAPD Primer code	Primer Sequence (5'-3')	Tm (°C)	No. of monomorphic bands	No. of polymorphic bands	Total no. of bands amplified	Size range (bp)
UBC-418	GAGGAAGCTT	36°C	1	0	1	800-1500
UBC-419	TACGTGCCCG	36°C	3	0	3	500-3000
UBC-420	GCAGGGTTCG	36°C	2	0	2	800-3000
UBC-421	ACGGCCCACC	36°C	2	0	2	500-3000
UBC-433	TCACGTGCCT	36°C	2	0	2	100-3000
UBC-438	AGACGGCCG	36°C	4	0	4	800-3000
UBC-449	GAGGTTCAAC	36°C	1	0	1	200-800
UBC-450	CGGAGAGCCA	36°C	3	0	3	100-2000
	Total		18	0	18	

Table 5.4 Details of the ISSR profile obtained from genomic DNA isolated from regenerated *M. acuminata* Colla var. Grand Naine (AAA) plantlets and the mother plant.

ISSR Primer code	Primer Sequence (5'-3')	Tm (°C)	No. of monomorphic bands	No. of polymorphic bands	Total no. of bands amplified	Size range (bp)
UBC-827	$(AC)_8G$	47.5°C	4	0	4	800-3500
UBC-829	$(TG)_8C$	47°C	4	0	4	800-2000
UBC-830	$(TG)_8G$	47°C	3	0	3	500-2000
UBC-834	$(AG)_8YT$	47°C	6	0	6	100-2000
UBC-840	$(GA)_8YT$	47°C	4	0	4	100-1000
UBC-843	$(CT)_8RA$	47°C	4	0	4	100-4500
UBC-844	$(CT)_8RC$	47.5°C	5	0	5	800-3000
UBC-845	$(CT)_8RG$	47°C	3	0	3	500-3500
	Total		33	0	33	

Where Y = C, T; R = A, G

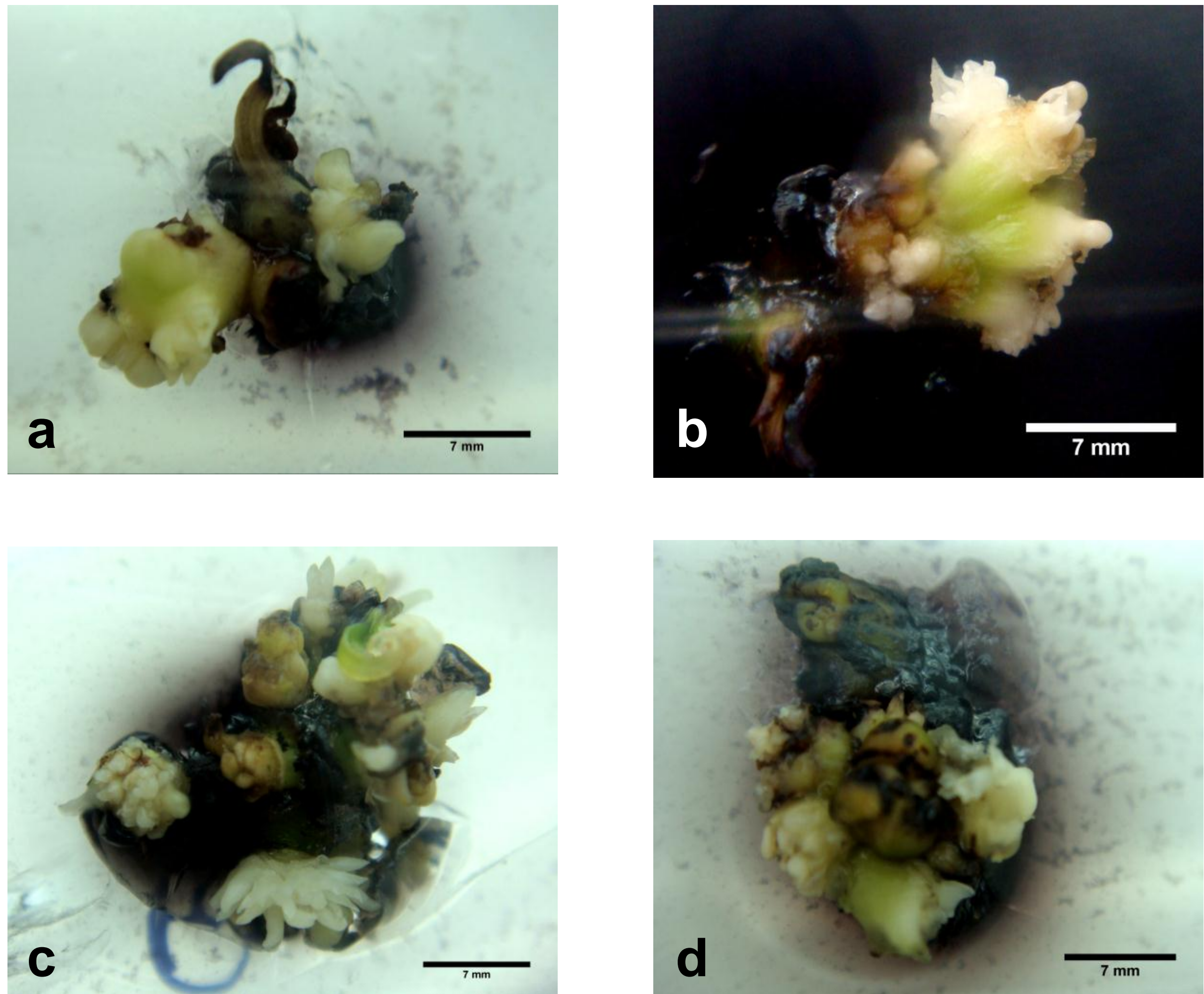

Figure 5.1 White bud-like structures (WBLS) observed from 4-5 weeks cultured male flowers of *M. acuminata* Colla var. Grand Naine (AAA): **a** – Appearance of WBLS in male flower cultured in MS + NAA (1 mg/L) after 5 weeks; **b** – Appearance of WBLS in male flower cultured in MS + BAP (1 mg/L) after 4 weeks; **c, d** – Appearance of numerous WBLS in male flowers cultured in MS + BAP (2 mg/L).

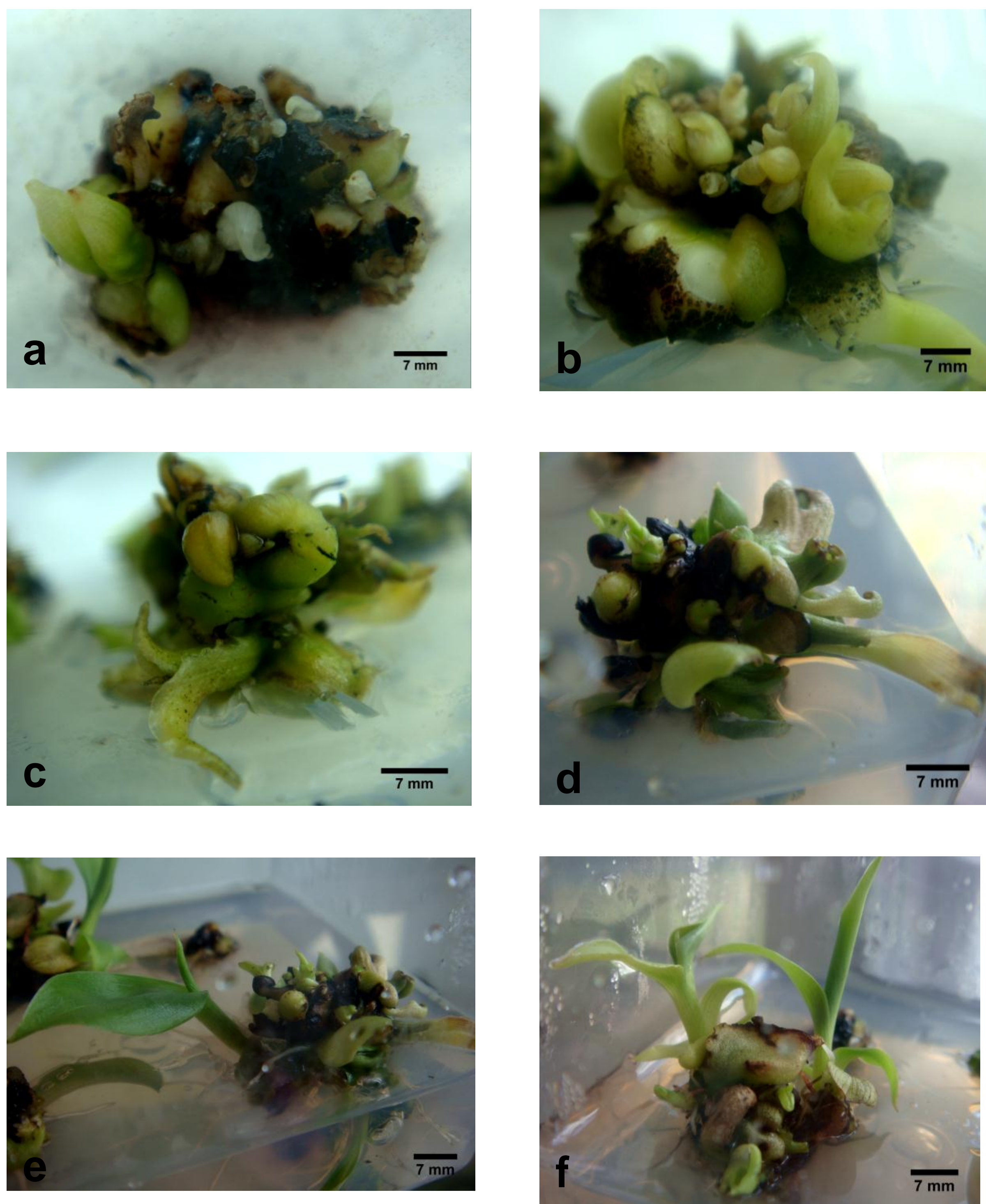

Figure 5.2 a-f– Different stages of shoot and leaf regeneration from WBLS derived from male flowers of *M. acuminata* Colla var. Grand Naine (AAA) after 15 weeks from initial culture.

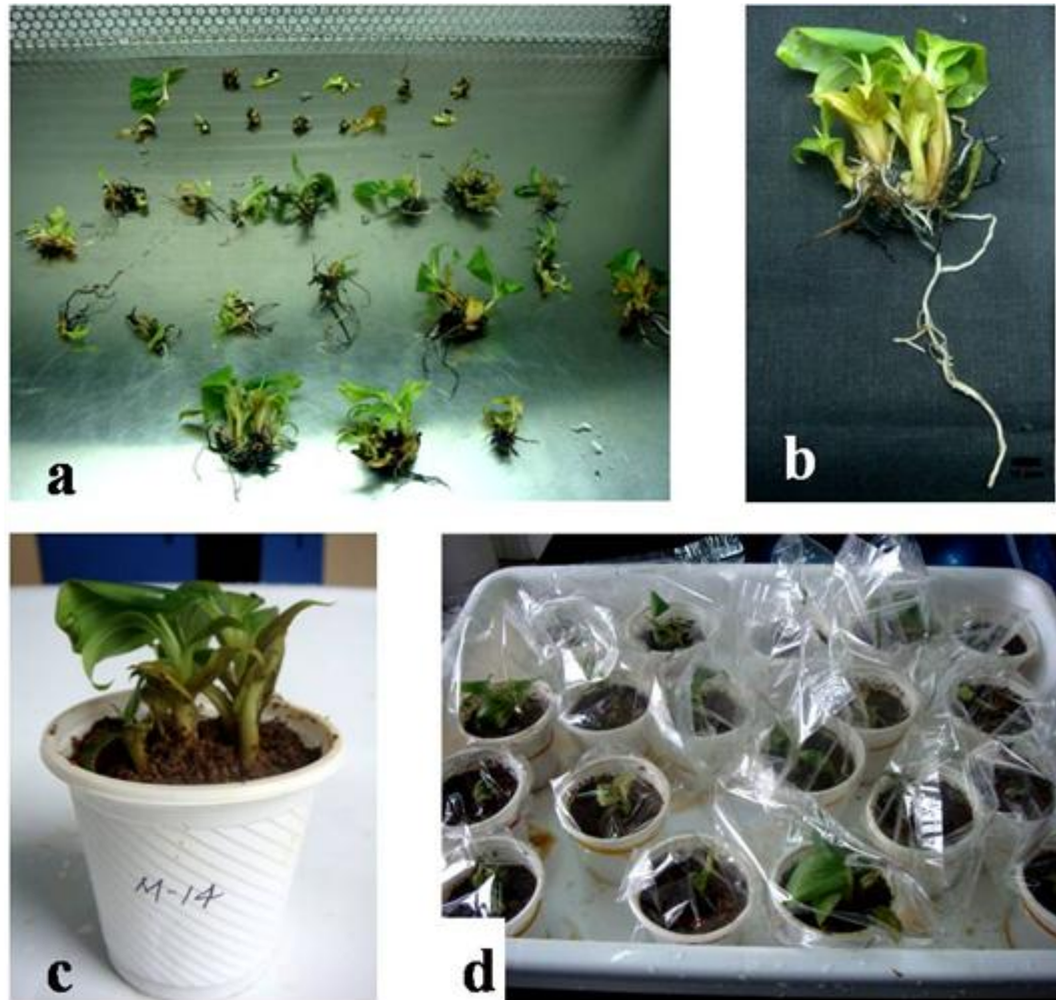

Figure 5.3 Fully developed regenerated plantlets of *M. acuminata* Colla var. Grand Naine (AAA): **a**– 27 plantlets obtained from one male bud from one experiment; **b** – A single plantlet showing multiple shoots and roots; **c** – Plantlet undergoing primary hardening in soil:sand (1:1); **d** – *In vitro* regenerated plantlets kept for primary hardening in soil:sand (1:1).

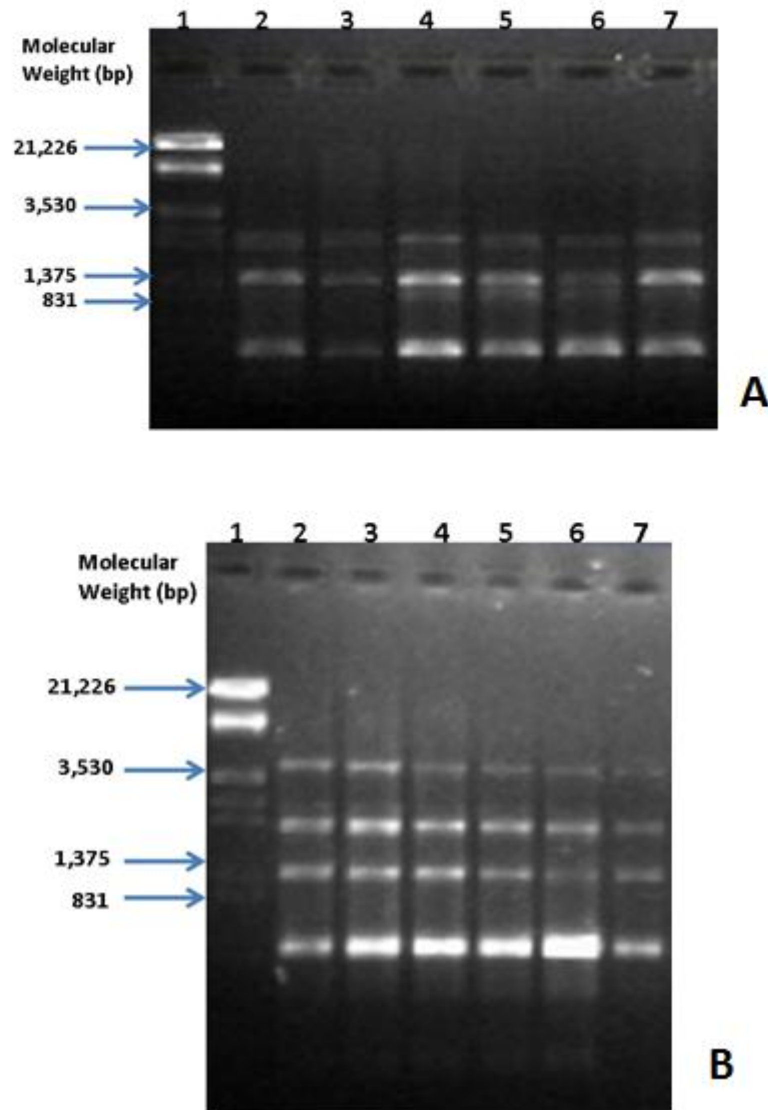

Figure 5.4 RAPD and ISSR profile of the *in vitro* regenerated plantlets derived from immature male flowers of *M. acuminata* Colla var. Grand Naine (AAA): **A** – Amplification with UBC RAPD Primer #450. **B** – Amplification with UBC ISSR Primer #843 Lane 1: lambda DNA HindIII marker, 2 donor plant, 3-7 regenerated plantlets.

Chapter 6

In vitro regeneration of *Musa acuminata* cv. Vaibalhla (AAA) using suckers as explant

CHAPTER 6

IN VITRO REGENERATION OF *MUSA ACUMINATA* CV. VAIBALHLA (AAA) USING SUCKERS AS EXPLANTS

6.1 Introduction

In the use of meristem-tip culture for plant multiplication, it is important to select the correct type of sucker for successful multiplication and subsequent plant regeneration. Sword suckers are recommended for banana cultivation by Hirimburegama and Gamage, 1996. The importance of selecting a suitable sucker for subsequent healthy plant growth and fruit production has also been reported in other cultivars such as in cv. Basrai (Nandwani *et al.*, 2000), *Musa sapientum* (Kalimuthu *et al.*, 2007), cv. Williams (Iqbal *et al.*, 2013). Hence, the use of sword suckers of *Musa acuminata* cv. Vaibalhla (AAA) as explants for *in vitro* regeneration was exhibited in this experiment. Bacterial contamination is a great problem for *in vitro* propagation of banana sucker explants. Although initially surface sterilization works, later on microbial contamination at the base of the explant is observed within 7 to 15 days after inoculation. Bacterial growth is also observed around the explants in the culture media. Huge numbers of explants are destroyed in the culture due to endogenous bacteria (Hadiuzzaman *et al.*, 2001).

6.2 Materials and Methods

6.2.1 Plant material

Sword suckers from selected mother plants of *M. acuminata* cv. Vaibalhla (AAA) maintained in the field gene bank of the Department of Biotechnology, Mizoram University, Aizawl were used as the explant source.

6.2.2 Optimization of sterilization procedure

Since suckers are present below the rhizosphere region, they contain many bacteria and viruses. It is necessary to follow an effective surface sterilization method. Sword suckers (Fig. 6.4 A) of selected elite plants of *M. acuminata* cv. Vaibalhla (AAA) were collected and the pseudo stem at the lower part containing meristem was selected as explant (Fig. 6.4 B). They were washed in running tap water for 20-25 minutes. The ensheathing leaf bases were removed from the pseudo stem, until the sucker becomes 5-6 cm in length, leaving the young leaves around the meristem. For surface-disinfection, the explant materials were treated with different solutions and combinations of 2% commercial fungicide Bavistin (BASF, India), 1% sodium hypochlorite (NaOCl) and 1% mercuric chloride ($HgCl_2$) solution for different set of timings (20 minutes, 30 minutes, 1 hour and overnight). Treatment with 2% bavistin was performed outside the LAF while further treatment with 1% NaOCl and 1% $HgCl_2$ were continued inside the LAF. After treatment with these sterilants, they were thoroughly washed 4-5 times with sterile distilled water. The outer leaf base were cut off again with sterile blade, until the sucker measured about 3 cm at the base and inoculated on MS medium supplemented with BAP (8 mg/L), NAA (2 mg/L) and AdS (50 mg/L). Each treatment consisted of 10 explants.

6.2.3 Standardization of media and plant growth regulators

For bud formation and shoot proliferation, the explants were inoculated aseptically in MS medium containing sucrose (30 g/L), myo-inositol (100 mg/L) and pH was adjusted to 5.5 – 5.8. The MS medium was variously supplemented with BAP (2 and 8 mg/L), NAA (0 and 2 mg/L), IAA (0 and 2 mg/L) and AdS (50 mg/L) in various

combinations. They were then solidified with 0.8% (w/v) agar and sterilized for 15 min at 121°C (15 lb psi pressure). Cultures were incubated at 25±1°C under 16:8 h light and dark photoperiod with light intensity of 55 μM/m^2/s using cool white fluorescent light (Bajaj Ltd., India) and 50-60% relative humidity (RH). All the cultures were subcultured into a fresh medium containing the same PGR every fourth week.

6.2.4 Control of phenolic exudation

A few days after culture, dark brownish phenolic exudations were observed from the explants which hamper the development and regeneration. Hence different concentrations of ascorbic acid (0, 25, 50, 75 and 100 mg/L) was supplemented in the media where explants are inoculated to control the exudation. In the present investigation, a batch of 15 explants each were cultured on MS medium supplemented with BAP (8 mg/L), NAA (2 mg/L) and AdS (50 mg/L) along with different concentrations of ascorbic acid in order to optimize the most effective concentration in controlling the exudation of phenolic compounds.

6.2.5 *In vitro* regeneration

The morphogenetic changes observed in the sucker explants inoculated in different combinations of BAP, NAA, IAA and AdS were recorded weekly. The frequency of explants swelling and bulging with tiny bud appearance was recorded. A batch of bulged explants were further cultured for shoot bud development, while another batch was divided into four segments and each segment was further inoculated onto a fresh medium consisting of the same PGRs for shoot bud development. The number of shoot bud per segment and subsequent number of shoots per bud was then recorded.

The evaluation of shoot length, root length and numbers per plantlet were recorded by sub-culturing in MS media with and without PGRs. Plantlets having fully expanded leaves and well-developed roots were transplanted for primary hardening in the laboratory containing sterilized soil: sand (1:1) and covered with polythene to maintain humidity. They were successfully transferred to greenhouse in pots containing farm yard manure: sand (1:1). For all experiments conducted, control explants were also cultured on MS media without any supplementation of plant growth regulator.

6.2.6 Data collection and statistical analysis

All experiments had three replicates per treatment. The experiments were repeated at least twice. The percentage data represented in the tables were arcsine transformed before being analyzed for significance using ANOVA (analysis of variance, $P < 0.05$). Further, the differences in means were contrasted using Duncan's new multiple range test following ANOVA. All statistical analysis was carried out using SPSS statistical software package version 16.0

6.3 Results

6.3.1 Optimization of sterilization procedure

Prior to inoculation, 10 sucker explants each were sterilized with five different combinations of 2% Bavistin, 1% NaOCl and 1% $HgCl_2$ solution for different set of timings (20 minutes, 30 minutes, 1 hour and overnight. Among the various treatments, the effective results in controlling contamination were obtained (Table 6.1). The least percentage of explant contamination (6.66) was seen when the explants were soaked overnight in 2% bavistin and kept for 30 minutes in 1% $HgCl_2$ (Fig. 6.1).

6.3.2 Control of phenolic exudation

For controlling the phenolic exudations, a batch of 15 explants each were cultured in MS medium supplemented with BAP (8 mg/L), NAA (2 mg/L) and AdS (50 mg/L) along with different concentrations of ascorbic acid (0, 25, 50, 75 and 100 mg/L). Within a period of 1 week and the level of exudation was observed (Table 6.2). Out of the five different concentrations tested, MS medium supplemented with 100 mg/L ascorbic acid could completely (100%) reduce the unwanted brownish exudations throughout the culture period (Fig. 6.2).

6.3.3 *In vitro* regeneration

In the present study, the successful *in vitro* regeneration of *M. acuminata* cv. Vaibalhla (AAA) plantlets were achieved using sucker explants. The explants were inoculated on MS medium with five different combinations of BAP, NAA, IAA and AdS along with 100 mg/L of ascorbic acid. Among the various treatments, the effective results obtained were recorded. After few days the explants swell and turn green, bulging from the base and producing tiny shoot buds within 2-8 weeks. The frequency of explant forming bud was found to be the highest (96.96%) in MS media supplemented with BAP (8 mg/L), NAA (2 mg/L) and AdS (50 mg/L) where buds initiated within 2-3 weeks (Table 6.3). From this stage onwards, the bulged explants were divided into two batches, one batch in which each explant was split into four segments (Fig 6.4 E) and subsequently transferred to a fresh medium, and another batch in which the explants were transferred as they are without splitting. New shoot buds (Fig 6.3) appeared from both batches within 3 weeks, and the number of shoot buds per explant was recorded. The highest formation of shoot buds were observed in

MS media supplemented with BAP (8 mg/L), NAA (2 mg/L) and AdS (50 mg/L). Splitted explants were found to give more shoot buds (6.66) compared to the un-splitted buds (3.33) (Table 6.3). The buds were transferred onto fresh MS media with the same PGR combinations where it further developed into shoots (Fig. 6.4 F, G). The frequency of shoot formation (97.22) as well as number of shoots per bud (5.66) were found to be the highest again in MS media supplemented with BAP (8 mg/L), NAA (2 mg/L) and AdS (50 mg/L) (Table 6.4).

The individual shoots were separated and sub-cultured in fresh media for elongation. MS medium supplemented with a combination of BAP (8 mg/L), NAA (2 mg/L) and AdS (50 mg/L) showed the maximum shoot length (14.63 cm) (Table 6.5). Appearance of leaves was observed from all the shoots developed by the 2nd week accompanied induction of roots. For the evaluation of rooting efficiency in different media the induced roots were cut off and the shoots were transferred to fresh media containing different PGRs. Within 1 week fresh roots appeared in varied numbers and the maximum number (8.33) and length (19.93 cm) of roots was observed in the MS basal medium without any PGRs (Fig 6.4 H).

The elongated shoots with roots were transferred to primary hardening. The well-developed healthy *in vitro* rooted plantlets were washed thoroughly in running tap water and planted in sterile soil: sand (1:1) (Fig 6.4 I). Plants were covered with transparent polyethylene bags to maintain adequate moisture for a week and transferred to the greenhouse (28°C day, 20°C night, 16 h day-length, and 70% humidity). After a week, the plastic covering was removed and the plantlets were maintained in the greenhouse in black plastic pots containing farm yard manure: sand

(1:1) (Fig. 6.4 J) until they were transplanted to the nursery. About 98% of the hardened plants survived in the nursery.

6.4 Discussion

In the present investigation, MS media supplemented with BAP (8 mg/L), NAA (2 mg/L) and AdS (50 mg/L) along with ascorbic acid (100 mg/L) proved to be the most effective combination for initiation and bud formation. The sucker explants were splitted after they start to bulge which gave rise to higher formation of buds as compared to the unsplitted ones. In most of the *in vitro* regeneration of *Musa* with sucker explants such as Shima banana (Buah *et al*., 1998), Basrai (Nandwani *et al*., 2000), *M. sapientum* (Kalimuthu *et al*., 2007), Williams (Iqbal *et al*., 2013), *M. sapientum* (Ramachandran and Amutha, 2013), PGRs such as BAP, IAA or NAA were utilized for initiation and shoot multiplication whereas in this experiment addition of AdS (50 mg/L) in all the media apart from the given PGRs favours the high proliferation of buds (6.66 buds per explant) and shoots (5.66 shoots per bud).

For the evaluation of rooting efficiency, the induced roots were cut off and the shoots were transferred to fresh media containing different PGRs. Within 1 week fresh roots appeared in varied numbers and the maximum number (8.33) and length (19.93 cm) of roots was observed in the MS basal medium without any PGRs (Table 6.5). This shows that addition of auxin or cytokinins did not promote any significant increase in the rooting frequency in agreement with the earlier observations in AAB cvs. Virupakshi and Sirumalai (Mahadev *et al*., 2011). Many of the previous reports on banana micropropagation used more than one type of media for initiation and multiplication (Cronauer and Krikorian, 1986; Jarret, 1986; Diniz *et al*., 1999;

Nauyen and Kozai, 2001; Krishnamoorthy *et al*., 2001; Kagera *et al*., 2004). In the present study a simple one-step protocol using MS with BAP, NAA and AdS for initiation, multiplication, and elongation of banana was used without the need to change PGR combinations. The protocol raised in the present attempt could be used for the massive *in vitro* production of the plantlets of *M.acuminata* cv. Vaibalhla (AAA).

Table 6.1 Effect of bavistin, sodium hypochlorite (NaOCl) and mercuric chloride ($HgCl_2$) treatment on the sterilization of sucker explants of *M. acuminata* cv. Vaibalhla (AAA) after 1 week of culture

Duration of sterilant used			Total no. of explants	Explants contaminated (%) (Mean±SE)
2.0 % Bavistin	1.0 % NaOCl	1.0 % $HgCl_2$		
-	20 min	-	10	96.66 ± 6.14a
1 hr.	20 min	-	10	76.66 ± 2.21bfg
overnight	30 min	-	10	60.00 ± 0.00cfg
1 hr.	-	20 min	10	43.33 ± 1.92dg
overnight	-	30 min	10	6.66 ± 6.14e

Medium: MS + BAP (8 mg/L) + NAA (20 mg/L) + AdS (50 mg/L). Mean (±) followed by the same letter(s) in each column are not significantly different at $P<0.05$ using Duncan's new multiple range test.

Table 6.2 Effect of ascorbic acid in controlling the phenolic exudation given out from sucker explants of *M. acuminata* cv. Vaibalhla (AAA) after 1 week.

Ascorbic acid (mg/L)	Total no. of explants	Explants without exudation (%) (Mean ± SE)
0.0	15	0.00 ± 0.00a
25.0	15	33.33 ± 4.22b
50.0	15	66.66 ± 4.22c
75.0	15	80.00 ± 0.00d
100.0	15	100.00 ± 0.00e

Medium: MS + BAP (8 mg/L) + NAA (2 mg/L) + AdS (50 mg/L). Mean (±) followed by the same letter(s) in each column are not significantly different at $P<0.05$ using Duncan's new multiple range test.

Table 6.3 Effect of MS basal medium supplemented with plant growth regulators on shoot bud formation from sucker explants of *M. acuminata* cv. Vaibalhla (AAA).

PGR (mg/L)				Time taken to form shoot buds (weeks)	Explant forming bud (%)	No. of buds/explant (Unsplitted) (Mean ± SE)	No. of buds/explant (Splitted) (Mean ± SE)
BAP	NAA	IAA	AdS				
0.0	0.0	0.0	0.0	0	0.00 ± 0.00a	0.00 ± 0.00a	0.00 ± 0.00a
2.0	2.0	-	50.0	5-6	87.87 ± 2.56bf	1.33 ± 0.33be	2.33 ± 0.33b
2.0	-	2.0	50.0	7-8	84.84 ± 2.56cf	0.66 ± 0.33ae	1.33 ± 0.33c
8.0	2.0	-	50.0	2-3	96.96 ± 5.85df	3.33 ± 0.33c	6.66 ± 0.33d
8.0	-	2.0	50.0	3-5	90.90 ± 7.47ef	2.33 ± 0.33d	3.33 ± 0.33e

Mean (±) followed by the same letter(s) in each column are not significantly different at P<0.05 using Duncan's new multiple range test.

Table 6.4 Effect of MS basal medium supplemented with plant growth regulators on shoot formation from buds derived from sucker explants of *M. acuminata* cv. Vaibalhla (AAA) after 3 weeks.

PGR (mg/L)				Shoot buds forming shoot (%)	No. of shoots/bud (Mean ± SE)
BAP	NAA	IAA	AdS		
2.0	2.0	-	50.0	86.10 ± 2.43a	1.66 ± 0.33ac
2.0	-	2.0	50.0	80.55 ± 1.96a	1.33 ± 0.33ac
8.0	2.0	-	50.0	97.22 ± 5.59b	5.66 ± 0.33b
8.0	-	2.0	50.0	88.88 ± 2.43a	2.66 ± 0.33a

Mean (±) followed by the same letter(s) in each column are not significantly different at P<0.05 using Duncan's new multiple range test.

Table 6.5 Effect of MS basal medium supplemented with and without plant growth regulators on the length of shoots and roots, and number of roots per plantlet derived from sucker explants of *M. acuminata* cv. Vaibalhla (AAA).

PGR (mg/L)				Length of shoots (cm) (Mean ± SE)	Length of roots (cm) (Mean ± SE)	No. of roots/plantlet (Mean ± SE)
BAP	NAA	IAA	AdS			
0.0	0.0	0.0	0.0	0.00 ± 0.00a	19.93 ± 0.08a	8.33 ± 0.33a
2.0	2.0	-	50.0	13.86 ± 0.41bf	18.66 ± 0.14be	4.33 ± 0.33bf
2.0	-	2.0	50.0	10.63 ± 0.29c	17.73 ± 0.12c	3.66 ± 0.33cf
8.0	2.0	-	50.0	14.63 ± 0.21df	19.70 ± 0.15a	6.33 ± 0.33dg
8.0	-	2.0	50.0	12.63 ± 0.20e	18.53 ± 0.08de	5.66 ± 0.33eg

Mean (±) followed by the same letter(s) in each column are not significantly different at $P<0.05$ using Duncan's new multiple range test.

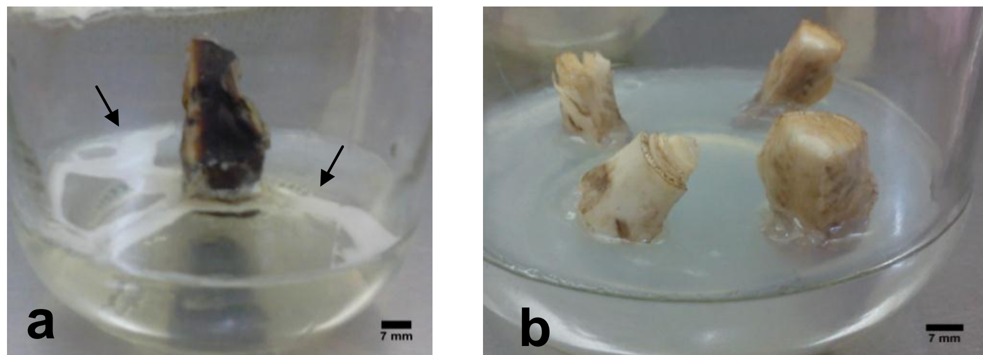

Figure 6.1 Effect of sodium hypochlorite (NaOCl), bavistin and mercuric chloride ($HgCl_2$) treatment on the sterilization of sucker explants of *M. acuminata* cv. Vaibalhla (AAA): **a** - Sucker explant with 1% NaOCl treatment for 20 minutes showing bacterial contamination after 5 days; **b** - Sucker explants treated with 2% bavistin overnight and 1% $HgCl_2$ for 30 minutes showing no sign of contamination after 5 days.

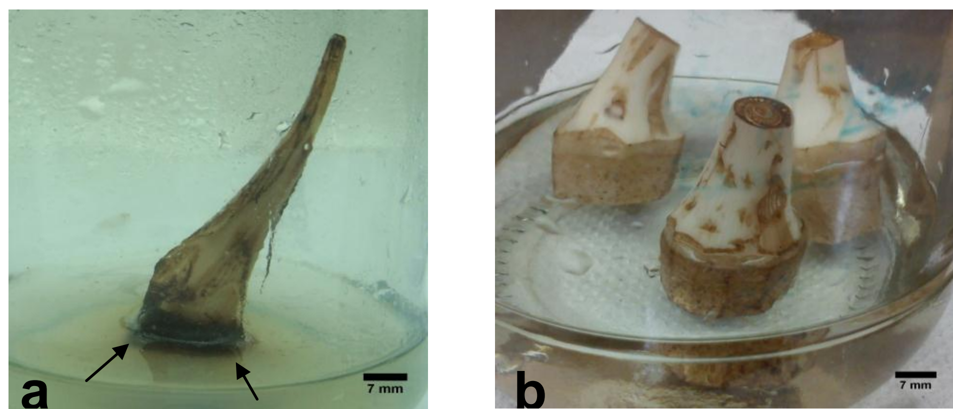

Figure 6.2 Effect of ascorbic acid in controlling the phenolic exudation given out from sucker explants of *M. acuminata* cv. Vaibalhla (AAA) after 1 week: **a** - Black phenolic exudation seen in sucker explant without ascorbic acid treated medium after 5 days; **b** - Sucker explants free from phenolic exudation with 100 mg/L ascorbic acid treated medium after 5 days.

Figure 6.3 Shoot bud development from one segment of the sucker explant of *M. acuminata* cv. Vaibalhla (AAA) cultured in MS + BAP (8 mg/L) + NAA (2 mg/L) + AdS (50 mg/L) + ascorbic acid (100 mg/L) after 2-3 weeks.

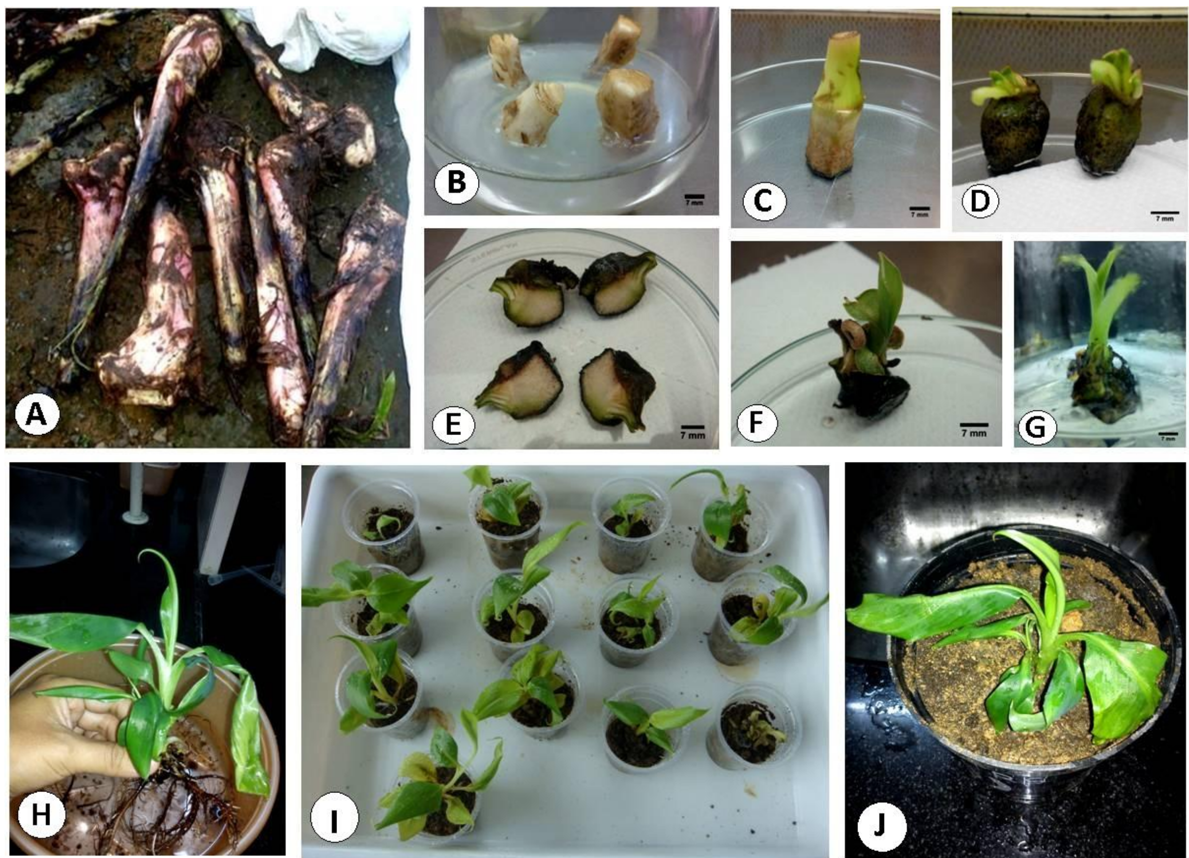

Figure 6.4 Different stages of the *in vitro* regeneration of *M. acuminata* cv. Vaibalhla (AAA) derived from sucker explants: **A** – Suckers, freshly removed from the mother plant; **B** – Explants aseptically cultured in MS media + PGRs; **C** –Explant showing green colouration after 10 days of culture; **D** – Explant showing bulging and shoot initiation in after 15 days; **E** – One sucker explant spilt to four segments after 15 days of culture; **F, G, H** – *In vitro* shoots and roots developing from one segment of the explant cultured in MS + BAP (8 mg/L) + NAA (2 mg/L) + AdS (50 mg/L) + ascorbic acid (100 mg/L); **I** – Primary hardening in soil:sand (1:1); **J** – Secondary hardened plant in FYM:sand (1:1).

Chapter 7

Genetic transformation of *Musa acuminata* cv. Vaibalhla (AAA) using *Agrobacterium tumefaciens*

CHAPTER 7

GENETIC TRANSFORMATION OF *MUSA ACUMINATA* CV. VAIBALHLA (AAA) USING *AGROBACTERIUM TUMEFACIENS*

7.1 Introduction

Development of new banana varieties through conventional breeding programs remains difficult because of sterility and polyploidy of most edible cultivars. Genetic transformation involving the introduction of agronomically important genes offers a viable means. Plant transformation mediated by *Agrobacterium tumefaciens*, a soil plant pathogenic bacterium has become the most used method for the introduction of foreign genes into plant cells and the subsequent regeneration of transgenic plants (Riva *et al*., 1998). *A. tumefaciens* has the exceptional ability to transfer a particular DNA segment (T-DNA) of the Ti plasmid into the nucleus of infected cells where it is then stably integrated into the host genome and transcribed, causing the crown gall disease (Nester *et al*., 1984; Binns and Thomashaw, 1988). Efficient *Agrobacterium*-mediated transformation requires optimal delivery of the T-DNA to regenerable cells of the explants. Wounding of explants allows *Agrobacterium* to better access plant cells as it stimulates the production of potent vir gene inducers, like phenolic substances and enhances the plant cell competence for transformation (Stachel *et al*., 1985; Shimoda *et al*., 1990; Bidney *et al*., 1992). Only plants with an appropriate wound response develop larger populations of wound adjacent competent cells for regeneration and transformation (Potrykus, 1991). Although excessive wounding is probably detrimental to stable transformation, the frequency of gene transfer via *Agrobacterium*-mediated transformation in recalcitrant species can be significantly enhanced by inducing wounds in the target tissue (Bidney *et al*., 1992). Wounding at

the regenerating sites before co-cultivation allowed better bacterial penetration into the regenerating cells of cotyledonary node explants, facilitating the accessibility of plant cells for *Agrobacterium* infection. Such mechanical wounding treatments greatly enhanced transformation efficiency in a number of plant species including recalcitrant grain legumes (Roome, 1992; Rohini *et al*., 2005; Supartana *et al*., 2006; Saini and Jaiwal, 2007).

Banana is a monocot crop that is mildly sensitive to salinity (Western Australian Agriculture Authority, 2008) and do not grow or fruit well in saline soils. Symptoms of salt damage include yellowing and death of the leaf margins and thin, deformed fruit (Crane and Balerdi, 1998). In the northeast region of Brazil, salinization in soils is one of the limiting factors for banana production (Gomes *et al*., 2004). The effect of salinity were demonstrated on both vegetative and chemical properties on some banana cultivars and a marked decrease was found in both vegetative growth and productivity (Israeli *et al*., 1986; Gomes *et al*., 2001; Mohamed, 2001; Abo El-Ez, 2003; Carmo *et al*., 2003; Gomes *et al*., 2004). Membrane proteins, such as vacuole Na+/H+ antiporters (NHX), play key roles in the tolerance of tissue to accumulate Na+ into vacuoles. In 1997, Nass *et al*. isolated the NHX1 gene from yeast and Gaxiola *et al*. (1999) cloned the AtNHX1 gene from *Arabidopsis thaliana* that was homologous to the NHX1 in yeast and restored the salt-sensitive yeast mutant phenotypes after introducing the AtNHX1 into yeast. The NHX1 encoded the Na+/H+ antiport in prevacuolar membrane. Numerous studies have shown that overexpression of the AtNHX1 gene confers salt tolerance to Arabidopsis plants and various other plant species (Apse *et al*., 1999; He *et al*., 2005; Leidi *et al*., 2010; Li *et al*., 2011). Furthermore, overexpession of AtNHX1 orthologs, such as OsNHX1 of *Oryza sativa*

L. (Chen *et al*., 2007), TaNHX2 of wheat (Jian *et al*., 2009), MdNHX1 of Malus (Li *et al*., 2010c) and AmNHX2 of *Ammopiptanthus mongolicus* (Wei *et al*., 2011), confers high salt tolerance to transgenic plants. In 2001, Zhang and Blumwald reported that the salt tolerance of tomato was improved by the transformation of AtNHX1.

Although initially *Agrobacterium*-mediated transformation was thought mainly applicable for dicotyledonous plants, understanding the biochemistry of infection process and finding out the need of acetosyringone for transfection, several monocots have been successfully transformed with *A. tumefaciens.* There have been a number of reports on the genetic transformation of genotype AAA banana accomplished either via *A. tumefaciens* (Sagi *et al*., 1995; May *et al*., 1995; Huang *et al*., 2007) or through biolistic methods. Up to now, there were no reports about introducing the AtNHX1 into *Musa* plants. In this study a series of experiments were conducted where explants of *M. acuminata* cv. Vaibalhla (AAA) were subjected to *A. tumefaciens*- mediated genetic transformation to investigate the capability of producing salt tolerant transgenic banana plants.

7.2 Materials and Methods

7.2.1 Explant preparation

Two types of explants were targeted for attempting transformation - immature male flower explants and sucker explants of *M. acuminata* cv. Vaibalhla (AAA).

Immature male flower explants were obtained from shortened male buds of 4-6 cm in length, where the male flowers are dissected aseptically after sterilization with

labolene for 30 minutes, 70% (v/v) ethanol for 5 minutes and 1% NaOCl for 5 minutes inside the LAF with a final sterile distilled water rinse. Individual male flowers of size 10mm – 20 mm were kept aside and divided into four batches which were then subjected to different pre-culture of 0, 7, 14 and 21 days in MS media supplemented with BAP (2 mg/L), NAA (0.5 mg/L) and ascorbic acid (75 mg/L).

For the sucker explants, sword suckers were collected and the pseudo stem at the lower part containing meristem was selected. It was washed in running tap water for 20-25 minutes. The ensheathing leaf bases were removed from the pseudo stem, until the sucker becomes 5-6 cm in length, leaving the young leaves around the meristem. The explant materials were then treated with aqueous solution of 2% bavistin overnight. These explants were then thoroughly rinsed with distilled water (4-5 times) and taken into the LAF. They were further treated with 1% $HgCl_2$ for 30 minutes and finally rinsed 4-5 times with sterile distilled water. The outer leaf base were cut off again with sterile blade leaving the explant to 4-5 cm in length, and kept separated for the experiment.

7.2.2 *Agrobacterium* strain

Agrobacterium tumefaciens strain EHA105 (Hood *et al.*, 1993) harboring the binary vector pCAMBIA2301 with NHX1 (Na+/H+ antiport) gene (isolated from *Arabidopsis thaliana*) inserted in the multiple cloning site (Courtesy: Dr. L. Sahoo, Indian Institute of Technology Guwahati, Guwahati, India) was used for the transformation experiment. *npt*II (neomycin phosphotransferase) was used as the selection marker and β-glucuronidase (GUS) gene (uidA) with an intron as the reporter gene within the T-DNA region driven by CaMV 35S promoter. Details of the

construct are given in Figure 7.1. The strain with the construct was maintained in the glycerol stock at – 80 °C.

7.2.3 Molecular confirmation of the *Agrobacterium* strain

7.2.3.1 Revival of the strain: The *A. tumefaciens* glycerol stock which was stored at -80 °C was revived by scraping off splinters of solid ice with a sterile toothpick or inoculation loop and plated onto a solid LB agar medium supplemented with 10 mg/L of rifampicin and 50 mg/L of kanamycin at 28°C.

7.2.3.2 Isolation of plasmid DNA: A small amount of freshly plated samples of the *A. tumefaciens* strain was picked from three colonies using sterile toothpick. A positive control was also prepared. Each sample was mixed with 30 µl of sterile MilliQ water in sterile eppendorf tubes which were thoroughly mixed by vortexing for 1-2 minutes. They were incubated in 100°C water-bath for 10 minutes, after which centrifugation was done at 10,000 rpm for 10 minutes. The supernatant serves as the template. The amount of isolated DNA per milligram of each sample was estimated by measuring absorbance at 260 nm and 280 nm using Bio-photometer plus (Eppendorf, Germany) according to the manufacturer's instruction. The purity of DNA was determined by calculating the ratio of absorbance at 260 nm to that of 280 nm. A 50 ng/µl DNA stock was prepared from the isolated DNA to be used for further experiments.

7.2.3.3 PCR confirmation: After preparation of the samples, PCR reactions were carried out in a 20 µl reaction mixture volume containing 50 ng of template DNA, 100 µM dNTP mix (Himedia, India), 0.4 µM of gene specific primer NHX1 (Fw: ATGTTGGATTCTCTAGTGTCG; Rv: TCAAGCCTTACTAAGATCAGG'), 1× Taq DNA polymerase buffer containing 15mM $MgCl_2$ (Sigma-Aldrich Pvt. Ltd.,

Bangalore) and 1.0 U of Taq polymerase (Sigma-Aldrich Pvt. Ltd., Bangalore). The PCR was carried out in a PTC-100TM thermal cycler (MJ research Inc, Waltham, Mass, USA) programmed with an initial denaturation of DNA at 95°C for 4 minutes, followed by 35 cycles of 95°C for 0.30 minutes, 58°C for 0.30 minutes and 72°C for 1:30 minutes followed by a final extension at 72°C for 10 minutes.The amplified products were resolved by electrophoresis on a 1.2% (w/v) agarose gel run in 1× TBE buffer and detected by ethidium bromide staining. The resulting fragments were scored under UV light using a gel documentation system (Bio-rad, Australia) and analysed with Quantity one-1D software (Bio-rad, Australia). A double-digested (EcoRI and HindIII) 1 Kb λ DNA ladder (Fermentas, USA) was used as molecular marker. All the PCR reactions were repeated at least twice to check the reproducibility.

7.2.4 Kanamycin sensitivity assay of the explants

The sensitivity test of selection agent were carried out in order to find the inhibitory concentration, which arrests the formation of buds from male flowers as well as suckers. The sensitivity of explants to kanamycin was determined by culturing the explants in bud inducing medium along with kanamycin. Prior to the transformation experiment, an effective concentration of kanamycin for the selection of transformed plants was determined by culturing non-transformed (control) explants on MS medium supplemented with BAP (2 mg/L), NAA (0.5 mg/L) and ascorbic acid (75 mg/L) for male flower explants and MS medium with BAP (8 mg/L), NAA (2 mg/L), AdS (50 mg/L) and ascorbic acid (100 mg/L) for sucker explants, containing different concentrations of kanamycin (0, 50, 75, 100 and 125 mg/L). The cultures were

transferred to a fresh medium containing the same level of antibiotic every 2 weeks and then scored for the frequency of bud formation.

7.2.5 Antibiotic assay for control of *Agrobacterium* overgrowth

Cefotaxime, augmentin and meropenem were used to prevent the over growth of *Agrobacterium*. To determine the optimum antibiotic concentration and combination that eliminates the *Agrobacterium*, the male flower explants were treated with the *A. tumefaciens* strain EHA105 harboring pCAMBIA2301AtNHX1 plasmid, and then subsequently placed in MS medium supplemented with BAP (2 mg/L), NAA (0.5 mg/L) and ascorbic acid (75 mg/L) containing different concentrations of cefotaxime (300, 400, 500 and 600 mg/L), augmentin (0 and 300 mg/L) and meropenem (0 and 35 mg/L) in single and in combinations. Treatments with the three antibiotics were also done on the sucker explants on MS medium with BAP (8 mg/L), NAA (2 mg/L), AdS (50 mg/L) and ascorbic acid (100 mg/L) containing different concentrations of cefotaxime (300, 500, 600, 700 and 800 mg/L), augmentin (0 and 300) and meropenem (0, 25 and 35 mg/L), in single and in combinations.

7.2.6 Preparation of the strain for transformation

A single bacterial colony of the *Agrobacterium* was inoculated into 25 ml of liquid AB minimal medium (Chilton *et al*., 1974) with appropriate antibiotics and grown overnight at 28°C on a rotary shaker at 180 rpm, until optical density at 600 nm reached to 0.8. The cells were collected by centrifuging at 5,000 rpm for 5 min, and then the pellet was resuspended in liquid MS basal medium (pH adjusted to 5.5) supplemented with 100 µM acetosyringone and used for inoculation.

7.2.7 Genetic Transformation

For each experiment, 30 explants were subjected to wounding treatment either by mechanical injury with needle or by sonication, and inoculated in bacterial suspension by occasional shaking for 30 min or by vacuum infiltration. The explants inoculated in bacterial suspension without prior wounding treatment were considered as control. After inoculation in all cases, explants were blotted on a sterile filter paper to remove excess liquid and co-cultivated for 3 days under dark condition at 22° C, in petri dishes lined with filter paper moistened with liquid MS basal medium supplemented with 100 µM acetosyringone.

7.2.8 Evaluation of the methods of injury techniques for the transformation efficiency

The three types of injury, in single and in combinations were all attempted on 30 male flower explants each, which were pre-cultured for 0, 7, 14 and 21 days.

The explants were wounded at the base by puncturing approximately 1 mm in depth with a sterile hypodermic needle (0.56 mm in diameter) prior to inoculation with *Agrobacterium* cell suspension. The puncturing was done at 1-2 points for one batch, and 4-5 points on other batch pre-cultured explants.

For sonication-assisted *agrobacterium tumefaciens* transformation (SAAT), the explants were immersed in 15 ml flat bottom glass culture tubes (Borosil, India) containing 7 ml of *Agrobacterium* cell suspension. The tubes were capped, placed in a float at the center of a bath-type sonicator (Telsonic ultrasonic TPC-40, Switzerland) and then subjected to ultrasound at a frequency of 30 kHz. The treatments differed as to sonication duration (30, 60 and 90 s). Following sonication, explants were removed

from the tubes, placed on sterile filter paper surface to blot off excess bacteria and then transferred to co-cultivation medium.

For vacuum infiltration (VI) experiment, it was combined with needle injury and SAAT to evaluate the effect in contrast to the use of these methods alone. Explants with or without puncturing and 30 s sonication treatments were placed in vacuum system consisting of a vacuum pump at 600 mm Hg (Rocker 400, Tarson, India) to which a desiccator was attached. Glass petri dishes containing explants immersed in *Agrobacterium* cell suspension were placed in the desiccator and vacuum was applied for duration of 15 minutes.

In all experiments, the frequency of transient GUS expression was analyzed after 3 days of co-cultivation. The optimal wounding, sonication and vacuum infiltration treatments were determined as the levels that led to a perceived increase in GUS positive foci in explants at the site of regeneration without any perceived decrease in explant viability. Control treatments consisted of explants either un-inoculated or inoculated with *Agrobacterium* without wounding, sonication and vacuum infiltration treatments.

7.2.9 Histochemical GUS assay

GUS activity was visualized using the histochemical assay (Jefferson, 1987). The explants were examined after 3 days of co-cultivation (Solleti *et al*., 2008). The explants were bleached with 100% ethanol for 24 hours prior to examination under a stereomicroscope. Transient expression of GUS was scored on a per explant basis by estimating the number of blue foci visible on the axillary region of each male flower explant. The blue foci were the discrete areas of cells with GUS activity

7.2.10 Explant recovery and regeneration

After 3 days of co-cultivation with *A. tumefaciens* harboring pCAMBIA2301AtNHX1 plasmid, the explants were washed five times with cefotaxime (2.0 mg/L) containing liquid MS basal medium and blotted dry on sterile filter paper and inoculated onto solid bud inducing medium containing kanamycin and antibiotics for 20 days with three rounds of subculture at an interval of 5, 7 and 8 days, respectively. The cultures were maintained at 25±1°C under 16:8 h light and dark photoperiod with light intensity of 55 μmol $m^{-2}s^{-1}$ using cool white fluorescent light (Bajaj Ltd., India) and 50-60% relative humidity (RH).

7.3 Results

7.3.1 Confirmation of presence of AtNHX1 gene in the *A. tumefaciens* strain EHA105

For the confirmation of the presence of AtNHX1 gene in the T-DNA of pCAMBIA plasmid a colony PCR was performed with AtNHX1 gene specific primer (Fw: ATGTTGGATTCTCTAGTGTCG; Rv: TCAAGCCTTACTAAGATCAGG'). The amplification of 1.6 kb fragments from EHA105pCAMBIA2301AtNHX1 clones confirmed the presence of NHX1 gene (Fig. 7.2). The entire three samples tested (lane 1, 2, 3) produced distinct band each at 1.6 Kb which was similar to the band found in positive control (lane 4).

7.3.2 Effect of kanamycin

Prior to transformation, an effective concentration of kanamycin for the selection of transformants was determined by culturing male flower explants on MS medium supplemented with BAP (2 mg/L), NAA (0.5 mg/L) and ascorbic acid (75 mg/L)

containing various concentrations of kanamycin (0, 50, 75, 100 and 125 mg/L) to determine the sub-lethal concentration (Table 7.1). Kanamycin concentration of 125 mg/L caused complete necrosis and inhibition of regeneration and growth of the explants leading to death by 6 weeks of culture. On the kanamycin free media, 76.66 % of the explants induced WBLS with an average of 4.03 WBLS/explant after 6 weeks of culture (Fig. 7.3 A). This result shows that 100 mg/L kanamycin is an effective selection marker for *M. acuminata* cv. Vaibalhla (AAA). However, for sucker explants, necrosis was not observed in any of the kanamycin concentration tested.

7.3.3 Effect of antibiotics

Influence of cefotaxime, augmentin and meropenem on the WBLS formation and subsequent development was checked by culturing male flower explants of *M. acuminata* cv. Vaibalhla (AAA) on MS medium supplemented with BAP (2 mg/L), NAA (0.5 mg/L) and ascorbic acid (75 mg/L) containing different concentrations of cefotaxime (300, 400, 500 and 600 mg/L), augmentin (0 and 300 mg/L) and meropenem (0 and 35 mg/L) in single and in combinations (Table 7.2). Of the different concentrations and combinations analysed, combination of cefotaxime 300 mg/L and augmentin 300 mg/L had less negative effect on WBLS formation from male flowers, but effective control against the growth of the *Agrobacterium* strain resulting in an average survival percentage of 82.04 (Fig. 7.3 B).

Similar influence of the three antibiotics were screened on the bud formation of the sucker explants of *M. acuminata* cv. Vaibalhla (AAA) by culturing them on MS medium supplemented with BAP (8 mg/L), NAA (2 mg/L), AdS (50 mg/L) and

ascorbic acid (100 mg/L) containing different concentrations of cefotaxime (300, 500, 600, 700 and 800 mg/L), augmentin (0 and 300 mg/L) and meropenem (0, 25 and 35 mg/L), in single and in combinations (Table 7.3). However, none of these concentrations and combinations could control the growth of *Agrobacterium* where pink colour bacterial contamination was observed (Fig. 7.4) which led to the discontinuation of transformation approach using sucker explants.

7.3.4 Effect of wounding and confirmation of putatively transformed explants using GUS assay

In the present investigation, male flower explants of *M. acuminata* cv. Vaibalhla (AAA) with different pre-culture days (0, 7, 14 and 21 days) were wounded via hypodermal needle, sonication-assisted *Agrobacterium*-mediated transformation (SAAT) and vacuum infiltration, and the most effective method of wounding suitable for this explant was studied and observed (Table 7.4, Table 7.5, Table 7.6 and Table 7.7). For determination of wounding efficiency, explants kept for histochemical GUS assay after 3 days co-cultivation showed blue colouration in putatively transformed explants.

In this experiment, 5 minutes of vacuum infiltration was combined with 1-2 needle point injury and with 30 seconds SAAT in order to increase the transformation efficiency on 0, 7, 14 and 21 days pre-cultured male flowers. Of the six experiments analysed using the wounding method combinatons, 30 seconds SAAT and 5 minutes of vacuum infiltration on 7 days pre-cultured male flowers produced the highest percentage of putative transformation (73.33 ± 4.22 %) (Table 7.5) showing GUS blue colouration (Fig. 7.5).

A number of male flower explants were subjected to needle point injury (1-2 points and 4-5 points) at the base where 4-5 needle point injury gave an average of 93.33% and 100% putative transformation on 14 days and 21 days pre-cultured male flower explants respectively confirmed with blue colouration when kept for GUS histochemical assay (Fig. 7.6 and Fig. 7.7).

The duration of sonication had some effect on the number of GUS positive banana male flowers. Duration of 30, 60 and 90 seconds were used for sonicating the male flower explants which were pre-cultured for 0, 7, 14 and 21 days in which putative transformations were observed in 14 and 21 days pre-cultured male flowers in rather low percentage (14.28 - 20%). (Table 7.6 and Table 7.7).

7.3.5 Regeneration of wounded explants

Three batch of transformation experiments were conducted utilizing the wounding method i.e. 4-5 needle point injury on 21 days pre-cultured male flower explant of *M. acuminata* cv. Vaibalha, co-cultivated with *A. tumefaciens* strain EHA105 harboring pCAMBIA2301AtNHX1 plasmid, kept for 3 days in dark with 100 µM acetosyringone, and cultured on bud inducing media (MS + BAP 2 mg/L + NAA 0.5 mg/L + ascorbic acid 75 mg/L + kanamycin 100 mg/L + cefotaxime 300 mg/L + augmentin 300 mg/L). 50-60 % GUS analysed explants were obtained. However, none of the explants were able to form buds and were unable to regenerate. (Table 7.8)

7.4 Discussion

Kanamycin was also used as selection marker during transformation of other banana cultivars such as cvs. Mpologoma and Nakitembe (East African highland banana – AAA) (Tripathi *et al*., 2008), cv. Gonja manjaya (AAB) (Tripathi *et al*., 2012) and in other plant species such as *Vigna radiata* (Jaiwal *et al*., 2001), *Vigna unguiculata* (Bakshi *et al*., 2011), *Parkia timoriana* (Thangjam and Sahoo, 2012).

Wounding of regenerating sites by a hypodermic needle is one of the commonly used methods for injuring explants. Wounding of the cotyledonary node explants of cowpea by a needle and co-cultivation with *A. tumefaciens* resulted in more efficient transient expression especially on needle wounded explants, mainly in terms of the percentage of explants showing GUS foci at the regenerating sites as compared to unwounded explants infected with *A. tumefaciens* (Bakshi *et al*., 2011). In the present study, wounding the 21 days pre-cultured immature male flowers of *M. acuminata* cv. Vaibalhla (AAA) with 4-5 needle points served to give the most efficient mode of injury which resulted in 100% putative transformation.

Recently, sonication has been used to enhance *Agrobacterium*- mediated transformation of many different plant species (Trick and Finer, 1997). SAAT consists of subjecting the target tissue to ultra sound and inoculating into *Agrobacterium* culture. The enhanced transformation efficiency using SAAT probably results from micro wounding, where the energy released by cavitation causes small wounds both on the surface of and deep within the target tissue, which facilitates *Agrobacterium* cells to enter into plant tissue. SAAT has been shown to provide efficient delivery of T-DNA into cells in a number of plants (Santarem *et al*.,

1998; Tang *et al*., 2001; Beranova *et al*., 2008), especially those that are typically more recalcitrant to *Agrobacterium*-mediated transformation (Trick and Finer, 1997). Zaragoza *et al*. (2004) produced herbicide resistant transgenic black locust plants by sonicating cotyledon explants for 1 min in *Agrobacterium* AGL1 carrying pTAB 16 plasmid suspensions. Trick and Finer (1998) and Santarem *et al*., (1998) successfully obtained transformed somatic embryos by sonicating immature soybean cotyledons with *Agrobacterium* cultures. The vacuum infiltration is one of the *Agrobacterium* based transformation systems, which has been successfully used to produce transgenic plants of bean (Liu *et al*., 2005), Arabidopsis (Clough and Bent, 1998), coffee (Canche-Moo *et al*., 2006), cotton (Leelavathi *et al*., 2004), and wheat (Cheng *et al*., 1997). This process increases gene transfer efficiency by improving penetration of *Agrobacterium* cells into the plant tissue layers. Trieu *et al*. (2000) obtained up to 76% transformation efficiency in *Medicago truncatula* through vacuum infiltration of seedlings for 3 min at 250 mm of Hg. Oliveira *et al*. (2009) used 2 seconds sonication combined with 10 minutes vacuum infiltration to produce transgenic citrus plants. Park *et al*. (2005) used 5 minutes sonication coupled with 5 minutes vacuum infiltration to transform radish plants. The association of sonication with vacuum infiltration dramatically improved the transformation efficiency in kidney bean (Liu *et al*., 2005).

For the present study, different durations of sonication (30, 60 and 90 s) alone and along with 5 mins of vacuum infiltration were applied on all pre-cultured explants. Single sonication treatments in all the pre-cultured explants did not show an effective result, but when combined with vacuum infiltration for 5 mins, the 7, 14 and 21 days pre-cultured immature male flower explants gave 73.33%, 66.66% and 71.42%

putative transformation respectively. However, vacuum infiltration combined with 1-2 needle point injury did not prove to be an effective mode of injury for immature male flowers of *M. acuminata* cv. Vaibalhla, since it resulted in putative transformation ranging between the frequencies of 33.33-38.88% for all the pre-cultured explants. However, the failure of the putatively transformed explants to regenerate into plantlets in the present study also shared the observations of Venkatachalam *et al*. 2011. The various conditions used for the regeneration of the putatively transformed explants will provide lead to future studies.

Table 7.1 Kanamycin sensitivity assay of male flowers of *M. acuminata* cv. Vaibalhla (AAA) on MS + BAP (2 mg/L) + NAA (0.5 m/l) + ascorbic acid (75 mg/L) after 6 weeks of culture.

Concentration (mg/L)	No. of explants cultured	Survival (%) (Mean ± SE)	No. of wbls/explant (Mean ± SE)
0.0	30	76.66 ± 2.21a	4.03 ± 0.06a
50.0	30	73.33 ± 2.21a	3.70 ± 0.17a
75.0	30	56.66 ±1.92b	3.13 ± 0.23b
100.0	30	36.66 ± 2.00c	1.60 ± 0.17c
125.0	30	0.00 ± 0.00d	0.00 ± 0.00d

Mean (±) followed by the same letter(s) in each column are not significantly different at P<0.05 using Duncan’s new multiple range test.

Table 7.2: Antibiotic assay of *Agrobacterium tumefaciens* strain EHA105 harboring pCAMBIA2301AtNHX1 plasmid inoculated on immature male flowers of*M. acuminata* cv. Vaibalhla (AAA) on MS + BAP (2 mg/L) + NAA (0.5 mg/L) + ascorbic acid (75 mg/L).

Antibiotic (mg/L)			Total no. of inoculated explants	Time (days) taken to show bacterial overgrowth	No. of explants showing bacterial overgrowth (Mean ± SE)	Survival (%) after 30 days (Mean ± SE)
Cef	Aug	Mer				
0.0	0.0	0.0	24	1	12 ± 0.00a	0.0 ± 0.00a
400.0	0.0	0.0	30	1-2	10 ± 0.00bg	0.0 ± 0.00a
500.0	0.0	0.0	30	3-4	10 ± 0.00cg	0.0 ± 0.00a
600.0	0.0	0.0	30	5-7	10 ± 0.00dg	0.0 ± 0.00a
300.0	300.0	0.0	39	14-18	2.33 ± 0.33e	82.04 ± 1.87b
500.0	0.0	35.0	30	6-7	10 ± 0.00fg	0.0 ± 0.00a

Mean (±) followed by the same letter(s) in each column are not significantly different at P<0.05 using Duncan’s new multiple range test.

Table 7.3 Antibiotic assay of *Agrobacterium tumefaciens* strain EHA105 harboring pCAMBIA2301AtNHX1 plasmid inoculated shoots (suckers) of *M.acuminata* cv. Vaibalhla (AAA) on MS + BAP (8 mg/L) + NAA (2 mg/L) + AdS(50 mg/L)) + ascorbic acid (100 mg/L).

Antibiotic (mg/L)			Total no. of inoculated explants	Time (days) taken to show bacterial overgrowth	No. of explants showing bacterial overgrowth (Mean ± SE)	Survival (%) after 30 days (Mean ± SE)
Cef	Aug	Mer				
0.0	0.0	0.0	15	1	5.00 ± 0.00	0.00 ± 0.00
500.0	0.0	0.0	15	1	5.00 ± 0.00	0.00 ± 0.00
700.0	0.0	0.0	15	1-2	5.00 ± 0.00	0.00 ± 0.00
500.0	300.0	0.0	15	2-3	5.00 ± 0.00	0.00 ± 0.00
700.0	300.0	0.0	15	2-3	5.00 ± 0.00	0.00 ± 0.00
300.0	0.0	25.0	15	2-3	5.00 ± 0.00	0.00 ± 0.00
500.0	0.0	35.0	15	2-3	5.00 ± 0.00	0.00 ± 0.00
600.0	0.0	35.0	15	2-3	5.00 ± 0.00	0.00 ± 0.00
700.0	0.0	35.0	15	2-3	5.00 ± 0.00	0.00 ± 0.00
800.0	0.0	35.0	15	2-3	5.00 ± 0.00	0.00 ± 0.00

Table 7.4 Effect of Injury on 0 day pre-cultured immature male flowers of *M. acuminata* cv. Vaibalhla (AAA) co-cultivated with *Agrobacterium tumefaciens* strain EHA105 harboring pCAMBIA2301AtNHX1 plasmid.

Type of injury							Un-inoculated			Inoculated with *Agrobacterium tumefaciens*		
1-2 NP	4-5 NP	30 s SAAT	60 s SAAT	90 s SAAT	1-2 NP + 5 min VI	30 s SAAT + 5 min VI	No. of explants	Percent of explants showing regeneration (%) (Mean ± SE)	Putative Transformation (%) (Mean ± SE)	No. of explants	Percent of explants showing regeneration (%) (Mean ± SE)	Putative transformation (%) (Mean ± SE)
-	-	-	-	-	-	-	30	66.66 ± 4.22a	0.00 ± 0.00	39	54.16 ± 2.41a	0.00 ± 0.00
+	-	-	-	-	-	-	30	60.00 ± 0.00b	0.00 ± 0.00	39	0.00 ± 0.00bi	0.00 ± 0.00
-	+	-	-	-	-	-	30	33.33 ± 4.22ci	0.00 ± 0.00	42	0.00 ± 0.00ci	0.00 ± 0.00
-	-	+	-	-	-	-	30	0.00 ± 0.00dj	0.00 ± 0.00	36	0.00 ± 0.00di	0.00 ± 0.00
-	-	-	+	-	-	-	30	0.00 ± 0.00ej	0.00 ± 0.00	36	0.00 ± 0.00ei	0.00 ± 0.00
-	-	-	-	+	-	-	30	0.00 ± 0.00fj	0.00 ± 0.00	36	0.00 ± 0.00fi	0.00 ± 0.00
-	-	-	-	-	+	-	30	33.33 ± 4.22gj	0.00 ± 0.00	36	0.00 ± 0.00gi	0.00 ± 0.00
-	-	-	-	-	-	+	30	0.00 ± 0.00hj	0.00 ± 0.00	30	0.00 ± 0.00hi	0.00 ± 0.00

NP: Needle Point, SAAT: Sonication Assisted *Agrobacterium tumefaciens* Transformation, VI: Vacuum Infiltration

Table 7.5 Effect of Injury on 7 days pre-cultured immature male flowers of *M. acuminata* cv. Vaibalhla (AAA) co-cultivated with *Agrobacterium tumefaciens* strain EHA105 harboring pCAMBIA2301AtNHX1 plasmid.

Type of injury							Un-inoculated			Inoculated with *Agrobacterium tumefaciens*		
1-2 NP	4-5 NP	30 s SAAT	60 s SAAT	90 s SAAT	1-2 NP + 5 min VI	30 s SAAT + 5 min VI	No. of explants	Percent of explants showing regeneration (%) (Mean ± SE)	Putative Transformation (%) (Mean ± SE)	No. of explants	Percent of explants showing regeneration (%) (Mean ± SE)	Putative transformation (%) (Mean ± SE)
-	-	-	-	-	-	-	30	66.66±4.22a	0.00±0.00	33	55.55±3.24a	0.00±0.00a
+	-	-	-	-	-	-	30	60.00±0.00a	0.00±0.00	36	33.33±2.85b	0.00±0.00a
-	+	-	-	-	-	-	30	20.00±0.00b	0.00±0.00	42	0.00±0.00ci	72.21±3.72be
-	-	+	-	-	-	-	30	0.00±0.00ch	0.00±0.00	36	0.00±0.00di	0.00±0.00a
-	-	-	+	-	-	-	30	0.00±0.00dh	0.00±0.00	36	0.00±0.00ei	0.00±0.00a
-	-	-	-	+	-	-	30	0.00±0.00eh	0.00±0.00	36	0.00±0.00fi	0.00±0.00a
-	-	-	-	-	+	-	30	40.00±0.00f	0.00±0.00	42	0.00±0.00gi	33.33±4.22c
-	-	-	-	-	-	+	30	0.00±0.00gh	0.00±0.00	33	0.00±0.00hi	73.33±4.22de

NP: Needle Point, SAAT: Sonication Assisted *Agrobacterium tumefaciens* Transformation, VI: Vacuum Infiltration

Table 7.6 Effect of Injury on 14 days pre-cultured immature male flowers of *M. acuminata* cv. Vaibalhla (AAA) co-cultivated with *Agrobacterium tumefaciens* strain EHA105 harboring pCAMBIA2301AtNHX1 plasmid.

Type of injury							Un-inoculated			Inoculated with *Agrobacterium tumefaciens*		
1-2 NP	4-5 NP	30 sec SAAT	60 sec SAAT	90 sec SAAT	1-2 NP + 5 min VI	30 sec SAAT + 5 min VI	No. of explants	Percent of explants showing regeneration (%) (Mean ± SE)	Putative Transformation (%) (Mean ± SE)	No. of explants	Percent of explants showing regeneration (%) (Mean ± SE)	Putative transformation (%) (Mean ± SE)
-	-	-	-	-	-	-	30	66.66±4.22a	0.00±0.00	33	61.10±3.24a	0.00±0.00a
+	-	-	-	-	-	-	30	60.00±0.00a	0.00±0.00	33	38.88±3.24b	0.00±0.00a
-	+	-	-	-	-	-	30	26.66±4.22bgh	0.00±0.00	39	0.00±0.00ci	93.33±8.85b
-	-	+	-	-	-	-	30	11.10±8.02cgh	0.00±0.00	36	0.00±0.00di	14.28±0.00chi
-	-	-	+	-	-	-	30	9.52±7.40dgh	0.00±0.00	36	0.00±0.00ei	20.00±0.00dhi
-	-	-	-	+	-	-	30	9.52±7.40egh	0.00±0.00	36	0.00±0.00fi	14.28±0.00ehi
-	-	-	-	-	+	-	30	53.33±3.84ag	0.00±0.00	39	0.00±0.00gi	33.33±0.00fi
-	-	-	-	-	-	+	30	11.10±8.02fgh	0.00±0.00	39	0.00±0.00hi	66.66±2.86g

NP: Needle Point, SAAT: Sonication Assisted *Agrobacterium tumefaciens* Transformation, VI: Vacuum Infiltration

Table 7.7 Effect of Injury on 21 days pre-cultured immature male flowers of *M. acuminata* cv. Vaibalhla (AAA) co-cultivated with *Agrobacterium tumefaciens* strain EHA105 harboring pCAMBIA2301AtNHX1 plasmid.

Type of injury							Un-inoculated			Inoculated with A*grobacterium tumefaciens*		
1-2 NP	4-5 NP	30 sec SAAT	60 sec SAAT	90 sec SAAT	1-2 NP + 5 min VI	30 sec SAAT + 5 min VI	No. of explants	Percent of explants showing regeneration (%) (Mean ± SE)	Putative Transformation (%) (Mean ± SE)	No. of explants	Percent of explants showing regeneration (%) (Mean ± SE)	Putative transformation (%) (Mean ± SE)
-	-	-	-	-	-	-	30	66.66±0.00a	0.00±0.00	33	55.55±3.24a	0.00±0.00a
+	-	-	-	-	-	-	30	60.00±0.00a	0.00±0.00	36	47.61±2.74b	0.00±0.00a
-	+	-	-	-	-	-	30	33.33±4.22b	0.00±0.00	39	0.00±0.00ci	100.00±0.00b
-	-	+	-	-	-	-	30	16.66±0.00cg	0.00±0.00	36	0.00±0.00di	19.04±2.12ch
-	-	-	+	-	-	-	30	14.28±0.00dg	0.00±0.00	36	0.00±0.00ei	20.00±0.00dh
-	-	-	-	+	-	-	30	12.50±0.00eg	0.00±0.00	36	0.00±0.00fi	19.04±4.76eh
-	-	-	-	-	+	-	30	53.33±3.84a	0.00±0.00	39	0.00±0.00gi	38.88±3.24f
-	-	-	-	-	-	+	30	16.66±0.00fg	0.00±0.00	39	0.00±0.00hi	71.42±0.00g

NP: Needle Point, SAAT: Sonication Assisted *Agrobacterium tumefaciens* Transformation, VI: Vacuum Infiltration

Table 7.8 Summary of transformation of 21 days pre-cultured immature male flower explants of *M. acuminata* cv. Vaibalhla (AAA) co-cultivated with *Agrobacterium tumefaciens* strain EHA105 harboring pCAMBIA2301AtNHX1 plasmid on MS + BAP (2 mg/L) + NAA (0.5 mg/L) + ascorbic acid (75 mg/L) + kanamycin (100 mg/L) + cefotaxime (300 mg/L) + augmentin (300 mg/L) with 4-5 needle point injury.

Experiment	No. of explants inoculated	No. of resistant explants showing shoot induction	Gus^+ analysed explants (%)	No. of plantlets regenerated per explant (%)
1	45	5	3/5 (60.00)	0 (0.00)
2	33	6	3/6 (50.00)	0 (0.00)
3	36	8	4/8 (50.00)	0 (0.00)
Total	114	19	10	0

Figure 7.1 Schematic construction of salt resistance gene AtNHX1 mobilized with *Agrobacterium tumefaciens* strain EHA105 harboring the binary vector pCAMBIA2301 containing *npt*II (neomycin phosphotransferase) as selectable marker and the β-glucuronidase (GUS) gene (uidA) with an intron as the reporter gene within the T-DNA region driven by CaMV 35S promoter. LB and RB: left border and right border of T-DNA region.

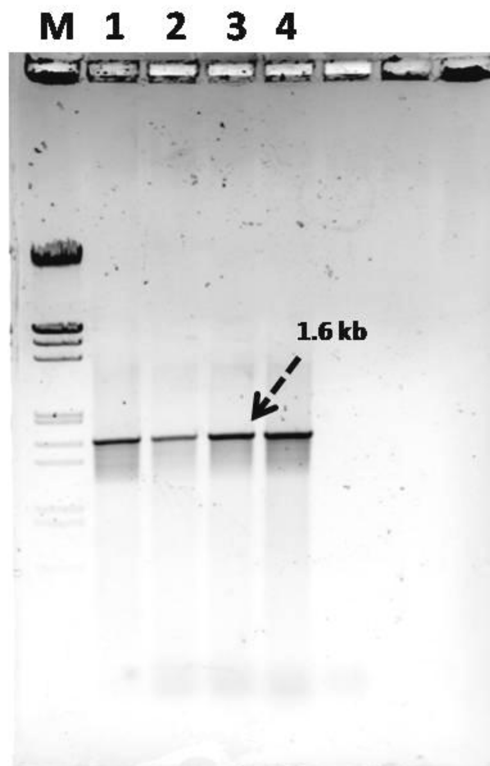

Figure 7.2 Confirmation of the presence of AtNHX1 gene in the T-DNA of the plasmid construct 35SpCAMBIA2301AtNHX1. M-Marker, 1- Sample, 2- Sample, 3- Sample, 4- AtNHX1 positive Control.

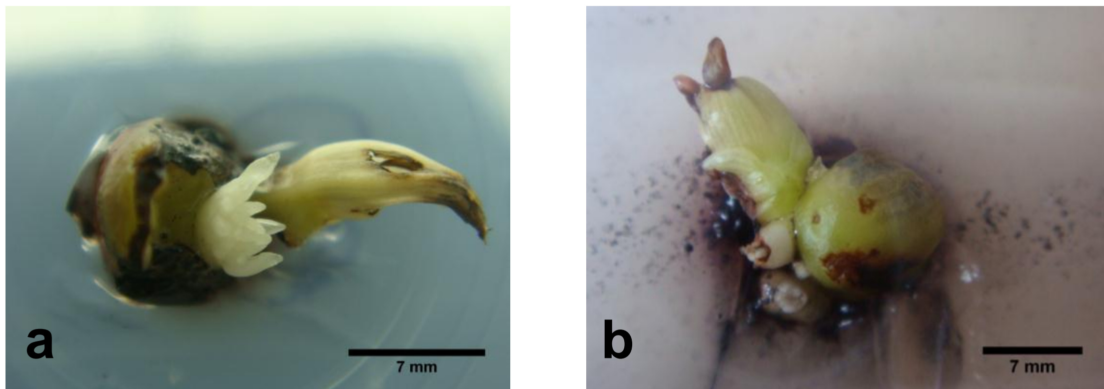

Figure 7.3 Kanamycin and antibiotic assay on the immature male flower explants of *M. acuminata* cv. Vaibalhla (AAA): **a**–Kanamycin assay on the immature male flower of *M. acuminata* cv. Vaibalhla (AAA) showing appearance of WBLS in male flower inoculated with 100 mg/L kanamycin after 6 weeks; **b**–Antibiotic assay of *Agrobacterium tumefaciens* strain EHA105 harboring pCAMBIA2301AtNHX1 plasmid inoculated male flowers of *Musa acuminata* cv. Vaibalhla (AAA) treated with cefotaxime (300 mg/L) + augmentin (300 mg/L) showing survival after 30 days.

Figure 7.4 Antibiotic assay on the sucker explants of *M. acuminata* cv. Vaibalhla (AAA):. Antibiotic assay of *Agrobacterium tumefaciens* strain EHA105 harboring pCAMBIA2301AtNHX1 plasmid inoculated suckers of *M. acuminata* cv. Vaibalhla (AAA) treated with cefotaxime (800 mg/L) + meropenem (35 mg/L) showing contamination after 2 days.

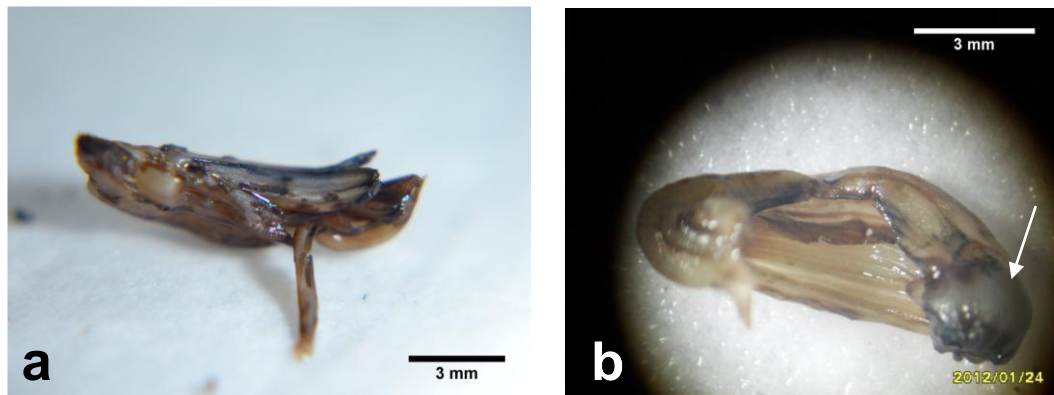

Figure 7.5 Histochemical GUS assay on 7 days pre-cultured immature male flower explants of *M. acuminata* cv. Vaibalhla (AAA)) injured with SAAT (30 secs) and Vacuum Infiltration (5 mins) inoculated with pCAMBIA2301AtNHX1 bacterial suspension culture kept for 3 days in dark with 100 µM acetosyringone: **a-** Control; **b** –Gus Positive explant.

Figure 7.6 Histochemical GUS assay on 14 days pre-cultured immature male flower explants of *M. acuminata* cv. Vaibalhla (AAA)) injured with 4-5 needle point injury inoculated with pCAMBIA2301AtNHX1 bacterial suspension culture kept for 3 days in dark with 100 µM acetosyringone.

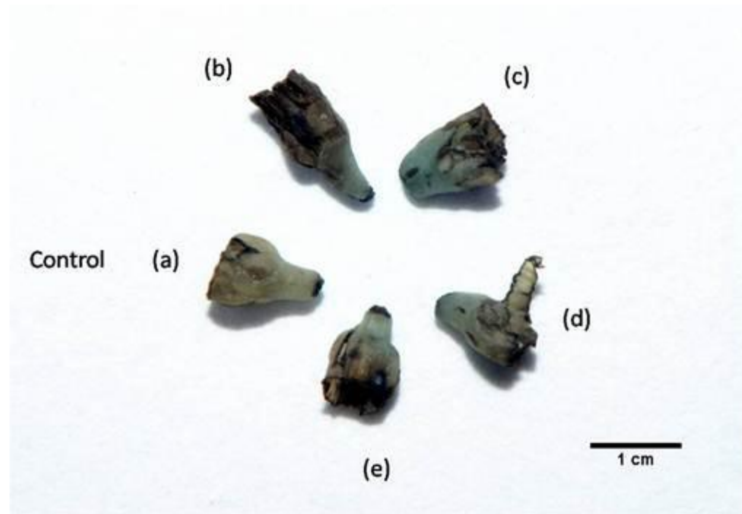

Figure 7.7 Histochemical GUS assay on 21 days pre-cultured immature male flower explants of *M. acuminata* cv. Vaibalhla (AAA) injured with 4-5 needle point injury inoculated with pCAMBIA2301AtNHX1 bacterial suspension culture kept for 3 days in dark with 100 µM acetosyringone: **a** – Control; **b-e** – Gus positive explants.

Chapter 8

Genetic transformation of *Musa acuminata* cv. Vaibalhla (AAA) using *Agrobacterium rhizogenes*

CHAPTER 8

GENETIC TRANSFORMATION OF *MUSA ACUMINATA* CV. VAIBALHLA (AAA) USING *AGROBACTERIUM RHIZOGENES*

8.1 Introduction

It is a well-known fact that diverse species of bacteria are capable of gene transfer to plants (Broothaerts *et al*., 2005) and *Agrobacterium* species are used widely for genetic modification in plants due to their gene integration capacity to the host genome. However, the use of *A. tumefaciens* is limited in banana plants since they are highly recalcitrant with no or very low regenerants (Ventkatachalam *et al*., 2011). An alternative to the limiting step of regeneration is the use of *A. rhizogenes*-mediated transformation leading to isolated hairy roots or composite plants having transformed roots and untransformed aerial part (Bosselut *et al*., 2011). Hairy roots are adventitious roots derived from cells transformed by the root inducing Ri plasmid of *A. rhizogenes* (Moore *et al*., 1979) and they grow in the absence of phytohormones. The hairy root harbours the T-DNA segment of the Ri plasmid within its nuclear genome (Chilton *et al*., 1982). *A. rhizogenes* has also been used for a few dicots such as coffee (Kumar *et al*., 2005) and broccoli with an antisense ACC oxidase gene (Henzi *et al*., 1999) and *Lithospermum erythrorhizon* with an uid A and *hpt*II marker (Yazaki *et al*., 1998). Other reports have also shown the use of *A. rhizogenes* for expression of the rol genes and to deliver foreign genes to susceptible plants (Christey *et al*., 1997).

Previous studies have indicated that *A. rhizogenes*-mediated transformation is a rapid and highly efficient system to investigate the function genes involved in root biology (Cho *et al*., 2000; Collier *et al*., 2005; Kereszt *et al*., 2007; Li *et al*., 2010a). However,

limited evidence supports the feasibility of using *A. rhizogenes*-mediated transformation to analyze the salinty tolerance in banana. In this study, NHX1 gene (isolated from *Vigna radiata*) was used for the *A. rhizogenes*-mediated transformation of *M. acuminata* cv. Vaibalhla (AAA).

8.2 Materials and Methods

8.2.1 Explant preparation

In vitro shoots were regenerated from immature male flowers of *Musa acuminata* cv. Vaibalhla (AAA) as discussed in Chapter 4. Established *in vitro* shoots were maintained on MS basal medium supplemented with BAP (2 mg/L), NAA (0.5 mg/L) and ascorbic acid (75 mg/L). Sucrose (30%) and myo-inositol (100 mg/L) were added in the media which was solidified with 0.8% (w/v) agar. The ph of the medium was adjusted to 5.5 – 5.8 before autoclaving at 121°C (15 lb psi pressure) for 15 minutes. Cultures were incubated at 25±1°C under 16:8 h light and dark photoperiod with light intensity of 55 µmol $m^{-2}s^{-1}$ using cool white fluorescent light (Bajaj Ltd., India) and 50-60% relative humidity (RH). Roots which appeared along with shoots were cut off prior to transformation experiment. Such clusters of regenerating tissues and shoot buds were used as starting explants for *Agrobacterium rhizogenes* transformation.

8.2.2 *Agrobacterium* strain

Agrobacterium rhizogenes strain A4 (Courtesy: Dr. L. Sahoo, Indian Institute of Technology Guwahati, Guwahati, India) was used for the transformation experiment. The gene of interest is NHX1 (Na+/H+ antiport) gene isolated from *Vigna radiata*. The A4 strain was mobilized with pCAMBIA2301:VrNHX1 plant binary construct using a modified protocol of Freeze-thaw method (Höfgen and Willmitzer, 1988).

*npt*II (neomycin phosphotransferase) was used as the selection marker and β-glucuronidase (GUS) gene (uidA) with an intron as the reporter gene within the T-DNA region driven by CaMV 35S promoter.

8.2.3 Mobilization of pCAMBIA2301:VrNHX1 plant binary construct to *Agrobacterium_rhizogenes* strain A4

A) Preparation of competent cells

An overnight culture of *A .rhizogenes* strain A4 was diluted with 100 ml of YMA (Yeast Mannitol Agar) liquid medium. After 3-4 hours, the logarithmically growing cells were analyzed where the optical density (OD) at 600 nm was 0.4 – 0.6. They were then centrifuged at 3000 g for 20 minutes at 4°C. The pellet was washed once in 5 ml precooled TE (10mM Tris-HCl, pH 7.5; 1mM EDTA) and resuspended in 10 ml fresh YMA medium. Aliquots of 500 μl were used directly for transformation or glycerol was added to final concentration of 20% , frozen in liquid nitrogen and stored at -80°C.

B) Mobilization

Stored cells were thawed on ice prior to mobilization. Competent agrobacteria were mixed with 0.5-1.0μg construct plasmid DNA. The cells were incubated successively 5 minutes on ice, 5 minutes in liquid nitrogen and 5 minutes at 37°C water-bath. After dilution in 1 ml of YMA medium the cells were shaken 2-4 hours at room temperature or 28°C. Aliquots of 200 μl were plated on solid YMA plates and incubated at 28°C for 24 hours. Bacterial colonies were picked and confirmed through colony PCR.

8.2.4 Molecular confirmation of the plasmid

The integrity of region of *nptII* and VrNHX1 within the *Agrobacterium* cells was confirmed through colony PCR using respective *npt*II and VrNHX1 gene specific oligonucleotide primers. A small amount of freshly plated samples of the *A. rhizogenes* strain was picked from a colony using sterile toothpick. 2 samples were prepared, each sample with a positive control and negative control. Samples were mixed with 30 µl of sterile MilliQ water in sterile eppendorf tubes which were thoroughly mixed by vortexing for 1-2 minutes. They were incubated in 100°C water-bath for 10 minutes followed by centrifugation at 10,000 rpm for 10 minutes. The supernatant serves as the template. The amount of isolated DNA per milligram of each sample was estimated by measuring absorbance at 260 nm and 280 nm using Bio-photometer plus (Eppendorf, Germany) according to the manufacturer's instruction. The purity of DNA was determined by calculating the ratio of absorbance at 260 nm to that of 280 nm. A 50 ng/µl DNA stock was prepared from the isolated plasmid DNA to be used for further experiments.

After preparation of the samples, PCR reactions were carried out in a 20 µl reaction mixture volume containing 50 ng of template DNA, 100 µM dNTP mix (Himedia, India), 0.4 µM of gene specific primers *npt*II (Fw: CCACCATGATATTCGGCAAC; Rv: GTGGAGAGGCTATTCGGCTA) and VrNHX1 (Fw: ATGTTGGATTCTCTAGTGTCG; Rv: TCAAGCCTTACTAAGATCAGG'), 1× Taq DNA polymerase buffer containing 15mM $MgCl_2$ (Sigma-Aldrich Pvt. Ltd., Bangalore) and 1.0 U of Taq polymerase (Sigma-Aldrich Pvt. Ltd, Bangalore). The PCR was carried out in a PTC-100TM thermal cycler (MJ research Inc, Waltham, Mass, USA) programmed with an initial denaturation of DNA at 95°C for 4 minutes,

followed by 35 cycles of 95°C for 0.30 minutes, 58°C for 0.30 minutes and 72°C for 1:30 minutes followed by a final extension at 72°C for 10 minutes.The amplified products were resolved by electrophoresis on a 1.2% (w/v) agarose gel run in 1× TBE buffer and detected by ethidium bromide staining. The resulting fragments were scored under UV light using a gel documentation system (Bio-rad, Australia) and analysed with Quantity one-1D software (Bio-rad, Australia). A double-digested (EcoRI and HindIII) 1 Kb λ DNA ladder (Fermentas, USA) was used as molecular marker. All the PCR reactions were repeated at least twice to check the reproducibility.

8.2.5 Kanamycin sensitivity assay of the explants

The sensitivity test of selection agent was carried out in order to find the inhibitory concentration, which arrests the formation of roots from cultured shoots. Prior to the transformation experiment, an effective concentration of kanamycin for the selection of transformed plants was determined by culturing non-transformed (control) *in vitro* shoot explants of *M. acuminata* cv. Vaibalhla (AAA) on MS medium supplemented with BAP (2 mg/L), NAA (0.5 mg/L) and ascorbic acid (75 mg/L) along with different concentrations of kanamycin (50, 100, 150 and 200 mg/L). The cultures were transferred to a fresh medium containing the same level of antibiotic every 2 weeks and then scored for the frequency of root formation.

8.2.6 Cefotaxime assay for control of *Agrobacterium* overgrowth

Cefotaxime was used to prevent the over growth of *Agrobacterium*. To determine the optimum cefotaxime concentration that eliminates the *Agrobacterium*, the aseptic 5 month old shoot explants derived from the male flowers of *M. acuminata* cv

Vaibalhla (AAA) were treated with the *Agrobacterium rhizogenes* strain A4 harbouring pCAMBIA2301VrNHX1 plasmid, and then subsequently placed in MS medium supplemented with BAP (2 mg/L), NAA (0.5 mg/L) and ascorbic acid (75 mg/L) containing different concentrations of cefotaxime (100, 200, 300, 400 and 500 mg/L).

8.2.7 Preparation of the strain for transformation

A single bacterial colony of the *A. rhizogenes* strain A4 harboring a binary vector pCAMBIA2301VrNHX1 which contains *npt*II (neomycin phosphotransferase) genes and β-glucuronidase (GUS) gene (uidA) with an intron in the coding region, both driven by CaMV 35S promoter was inoculated into 25 ml of liquid YMA medium and grown overnight at 28°C on a rotary shaker at 180 rpm, until optical density at 600 nm reached to 0.8. The cells were collected by centrifuging at 5,000 rpm for 5 min, and then the pellet was resuspended in liquid MS basal medium (pH adjusted to 5.5) supplemented with 100 µM acetosyringone and used for inoculation.

8.2.8 Genetic Transformation

For the transformation experiments the 5 month old *in vitro* regenerated shoots derived from male flowers of *M. acuminata* cv. Vaibalhla (AAA) were collected. The existing roots were cut off using sterile blade and the basal part of the shoots were immersed into the resuspended pellets of the *Agrobacterium* with occasional shaking. Inoculated explants were blotted on sterile filter paper and co-cultured in solid MS basal medium supplemented with 100 µM acetosyringone. The co-cultures were maintained in dark. After co-cultivation, the explants were washed three to four times with liquid MS basal medium and blotted dry on sterile filter paper. The washed

explants were cultured on MS basal medium and maintained at 25±1°C under 16:8 h light and dark photoperiod with light intensity of 55 µmol $m^{-2}s^{-1}$ using cool white fluorescent light (Bajaj Ltd., India) and 50-60% relative humidity (RH). Different experiments were conducted independently to evaluate the factors influencing the transformation efficiency, as follows: (1) 36 *in vitro* shoot explants were inoculated for 0, 10, 20, 30 and 40 minutes with *Agrobacterium* suspension to determine the optimum duration of inoculation peroid; (2) 33 explants were co-cultivated with *Agrobacterium* for 0, 1, 2, 3 and 4 days in dark to determine the optimum duration for co-culture.

8.2.9 Regeneration and histochemical GUS assay of putatively transformed plants

GUS activity was visualized using the histochemical assay (Jefferson, 1987). Transient expression was examined after roots were developed from the inoculated shoots of *M. acuminata* cv. Vaibalhla (AAA). The roots were cut off and immersed in GUS substrate solution for 24 hours at 37°C. Following incubation, roots were observed under stereomicroscope. The efficiency was calculated by taking percentage of the GUS positive roots for evaluating the optimal condition of transformation. Transient expression of GUS was scored on a per explant basis by estimating the number of blue foci visible on the axillary region of each root. The blue foci were the discrete areas of cells with GUS activity.

Under the optimized transformation conditions, 3 independent experiments were conducted separately with 15, 20 and 25 explants, respectively, for the transformation assay. The co-cultured explants were cultured on semi solid MS basal medium containing 150 mg/L kanamycin and 400 mg/L cefotaxime. The explants were transferred onto fresh medium containing the same levels of anibiotics every 2 weeks,

until roots developed and attained the length of 5-7 cm. Histochemical GUS activity was carried out on the regenerated roots by cutting 1-2 cm basal sections. The frequencies of putatively transformed plants were calculated based on the numbers and percentages of GUS positive (GUS^+) roots, which showed any GUS activity.

8.2.10 Molecular analysis of the putative transformants

For the PCR screening of the putative transformants, total genomic DNA was extracted from fresh roots of putatively transformed and non-transformed (control) plants of *M. acuminata* cv. Vaibalhla (AAA) by using Himedia DNA isolation kit (HiPurA™ Plant Genomic DNA Miniprep Purification Kit) and screened by PCR for the presence of the *npt*II gene. The 540 bp region of *npt*II was amplified using 20 mers (Fw: CCACCATGATATTCGGCAAC; Rv: GTGGAGAGGCTATTCGGCTA) and 1.6 kb region of the NHX1 (Fw: ATGTTGGATTCTCTAGTGTCG; Rv: TCAAGCCTTACTAAGATCAGG) gene specific oligonucleotide primers. The amplification reaction was carried out under the following conditions: 95°C for 3 minutes (1cycle), 95°C for 1 minute (denaturation), 58°C for 0.30 minute (annealing), 72°C for 1 minute (extension) for 35 cycles followed by the final extension at 72°C for 10 minutes (1 cycle). PCR was performed in a 20 µl reaction mixture volume containing ~100 ng of purified genomic DNA, 100 µM dNTP mix (Himedia, India), 0.4 µM of gene specific primer *npt*II, 1× Taq DNA polymerase buffer containing 15mM $MgCl_2$ (Sigma-Aldrich Pvt. Ltd., Bangalore) and 1.0 U of Taq polymerase (Sigma-Aldrich Pvt. Ltd., Bangalore). To ensure that reagents were not contaminated, DNA from non-transformed (control) plants was included in the experiments.The amplified products were resolved by electrophoresis on 1% agarose gel and visualized by ethidium bromide staining .The resulting fragments were scored under UV light

using a gel documentation system (Bio-rad, Australia) and analysed with Quantity one-1D software (Bio-rad, Australia). A double-digested (EcoRI and HindIII) 1 Kb λ DNA ladder (Fermentas, USA) was used as molecular marker. All the PCR reactions were repeated at least twice to check the reproducibility.

8.3 Results

8.3.1 Confirmation of presence of *npt*II and VrNHX1 gene in the *A. rhizogenes* strain A4

For the confirmation of the presence of *npt*II and VrNHX1 gene in the T-DNA of pCAMBIA plasmid a colony PCR was conducted with the *npt*II (Fw: CCACCATGATATTCGGCAAC; Rv: GTGGAGAGGCTATTCGGCTA) and NHX1 (Fw: ATGTTGGATTCTCTAGTGTCG; Rv: TCAAGCCTTACTAAGATCAGG') gene specific primers. The plant binary construct pCAMBIA2301:VrNHX1 was successfully mobilized to *A. rhizogenes* strain A4 by modified Freeze-thaw method and mobilization was confirmed by colony PCR. The amplification of 540 bp and 1.6 kb fragments from A4pCAMBIA2301VrNHX1 clones were confirming the presence of *npt*II and NHX1 genes respectively (Fig. 8.2). The schematic construction of VrNHX1 mobilized with *Agrobacterium tumefaciens* strain EHA105 harboring the binary vector pCAMBIA2301 containing *npt*II (neomycin phosphotransferase) as selectable marker and the β-glucuronidase (GUS) gene (uidA) with an intron as the reporter gene within the T-DNA region driven by CaMV 35S promoter is given in Figure 8.1.

8.3.2 Effect of kanamycin

Prior to transformation, an effective concentration of kanamycin for the selection of transformants was determined by culturing *in vitro* shoot explants on MS medium supplemented with BAP (2 mg/L), NAA (0.5 mg/L) and ascorbic acid (75 mg/L) containing various concentrations of kanamycin (50, 100, 150 and 200 mg/L) to determine the sub-lethal concentration. Kanamycin concentration of 150 mg/L caused complete necrosis and inhibition of regeneration and growth of the explants leading to death by 6 weeks of culture (Table 8.1). On the kanamycin free media, 100% of the explants induced roots with an average of 7.46 roots per explant after 5 weeks of culture. This result shows that 150 mg/L kanamycin is an effective selection marker for root development from *in vitro* shoots of *M. acuminata* cv. Vaibalhla (AAA).

8.3.3 Effect of cefotaxime

Influence of cefotaxime on the root formation was checked by culturing *in vitro* shoot explants of *M. acuminata* cv. Vaibalhla (AAA) on MS medium supplemented with BAP (2 mg/L), NAA (0.5 mg/L) and ascorbic acid (75 mg/L) containing different concentrations of cefotaxime (100, 200, 300, 400 and 500 mg/L) (Table 8.2). Of the different concentrations analysed, cefotaxime 400 mg/L had less negative effect on root formation from *in vitro* shoots, but effective control against the growth of the *Agrobacterium* strain resulting in an average survival percentage of 93.33.

8.3.4 Optimization of inoculation period and co-culture duration

The explant inoculation efficiency varied from placing the drops of bacterial suspension to different times of incubation, within 10 to 30 minutes. In this study, the longer the time of inoculation the percentage of GUS^+ plants decreases. It can be

concluded that the time of inoculation for 30 minutes is the optimum for getting a good percentage of GUS^+ roots. (Table 8.3).The extension of co-culture period of the explants with *Agrobacterium* upto 2 days increased the putative transformation frequencies. Further extension in co-cultivation time decreased the transformation frequency resulting in bacterial overgrowth (Table 8.4).

8.3.5 Regeneration and confirmation of putatively transformed roots

Following the optimized conditions of co-culture, the *in vitro* shoot explants derived from male flowers of *M. acuminata* cv. Vaibalhla (AAA) were cultured on MS basal medium containing 75 mg/L ascorbic acid, 150 mg/L kanamycin and 400 mg/L cefotaxime for root regeneration. After 2 days of culture, new roots start to appear. The resistant roots formed were having lateral roots of uniform length (Fig. 8.6). Within 5 weeks of culture, out of 3 experiments, an average of 11.33 kanamycin resistant roots were obtained (Table 8.5). The explants were transferred onto fresh medium containing the same levels of antibiotics every 2 weeks until roots attained the length of 5-7 cm. Histochemical GUS activity in the regenerated roots were detected with the appearance of blue colour on the root tips. Putatively transformed root showed pale blue colour GUS expression in 30 minutes inoculated explant (Fig. 8.3), and also in roots co-cultivated for 2 days (Fig. 8.4). With a combination of 30 minutes inoculation and 2 days co-cultivation, intense blue GUS expression was observed (Fig. 8.5). Overall, a total of 17 GUS^+ roots were obtained from the 3 different independent transformation experiments. The PCR analysis of the regenerated kanamycin resistant GUS^+ roots showed amplification on the 540 bp fragments corresponding to the *npt*II gene, indicating the presence of transgenes but no amplification in the control non-transformed roots (Fig 8.7). However, not all the

34 regenerated roots showed the presence of the fragment. The frequency of transformation revealed by the percentage (%) PCR + roots of the total number of explants varied from 12.00 to 20.00%.

8.4 Discussion

The use of organized cultures for transformation is appealing because of its relatively short regeneration time. May *et al* (1995) produced transgenic Cavendish cv. Grand Naine by co-cultivating wounded meristems with *A. tumefaciens*. However, some workers consider this technique is of limited value because of the potential generation of chimeric plants (Becker *et al*., 2000) probably occurring as `escapes' on the selection medium. Nevertheless, in the present study, the repeated culture of the putative transformants resulted in the survival of only complete transformants and no escapes were encountered indicating the feasibility of organized structures such as shoot buds and reap the advantage of faster results.

In previous *A. rhizogenes*-mediated genetic transformation of Silk banana (AAB), kanamycin (200 mg/L) was used for eradicating *Agrobacterium* overgrowth whereas in the present investigation, cefotaxime (400 mg/L) served the purpose. The present study appears to be the first report on banana genotype AAA in obtaining transient transformation using *A. rhizogenes* and in successfully transforming salt tolerant VrNHX1 gene to hairy roots resulting in formation of banana composite plants.

Table 8.1 Kanamycin assay on 5 months old *in vitro* regenerated shoots derived from immature male flower explants of *M. acuminata* cv. Vaibalhla (AAA) on MS + BAP (2 mg/L) + NAA (0.5 m/l) + ascorbic acid (75 mg/L) after 5 weeks of culture.

Concentration (mg/L)	No. of shoots inoculated	Survival (%) (Mean ± SE)	No. of roots/shoot (Mean ± SE)
0.0	15	100 ± 0.00a	7.46 ± 0.17a
50.0	15	100 ± 0.00a	7.00 ± 0.11a
100.0	15	53.33 ± 3.84b	3.66 ± 0.40b
150.0	15	33.33 ± 4.22c	0.53 ± 0.17c
200.0	15	0.00 ± 0.00d	0.00 ± 0.00d

Mean (±) followed by the same letter(s) in each column are not significantly different at P<0.05 using Duncan's new multiple range test.

Table 8.2 Antibiotic (Cefotaxime) assay on 5 months old *in vitro* regenerated shoots derived from immature male flower explants of *M. acuminata* cv. Vaibalhla (AAA) inoculated with *Agrobacterium rhizogenes* strain A4 harboring pCAMBIA2301VrNHX1 on MS + BAP (2 mg/L) + NAA (0.5 mg/L) + ascorbic acid (75 mg/L).

Concentration (mg/L)	No. of shoots inoculated	Time (days) taken to show bacterial overgrowth	No. of explants showing bacterial overgrowth (Mean ± SE)	Survival (%) after 30 days (Mean ± SE)
0.0	15	1	5.00 ± 0.00a	0.00 ± 0.00a
100.0	15	1	5.00 ± 0.00a	0.00 ± 0.00a
200.0	15	3-5	4.33 ± 0.33a	13.33 ± 8.85a
300.0	15	3-5	2.33 ± 0.33b	53.33 ± 3.84b
400.0	15	7-10	0.33 ± 0.33ce	93.33 ± 8.85ce
500.0	15	7-10	0.33 ± 0.33de	93.33 ± 8.85de

Mean (±) followed by the same letter(s) in each column are not significantly different at P<0.05 using Duncan's new multiple range test.

Table 8.3 Effect of inoculation time on the efficiency of *Agrobacterium rhizogenes* strain A4 harboring pCAMBIA2301VrNHX1 transformation of *in vitro* regenerated shoots derived from immature male flower explants of *M. acuminata* cv. Vaibalhla (AAA).

Inoculation period (min)	No. of explants	Putative transformation (%)[a]
0	36	0.00 ± 0.00a
10	36	19.44 ± 1.97b
20	36	30.55 ± 1.75cg
30	36	44.44 ± 1.60dfg
40	36	36.10 ± 1.64efg

Table 8.4 Effect of co-cultivation duration on the efficiency of *Agrobacterium rhizogenes* strain A4 harboring pCAMBIA2301VrNHX1 transformation of *in vitro* regenerated shoots derived from immature male flower explants of *M. acuminata* cv. Vaibalhla (AAA).

Co-cultivation duration (days)	No. of explants	Putative transformation (%)[a]
0	33	0.00 ± 0.00a
1	33	12.12 ± 2.56bfg
2	33	39.39 ± 1.76c
3	33	15.15 ± 2.56dfg
4	33	18.18 ± 4.03eg

Table 8.5 Summary of transformation of *in vitro* regenerated shoot explants derived from immature male flowers of *M. acuminata* cv. Vaibalhla (AAA) for hairy root formation co-cultivated with *Agrobacterium rhizogenes* strain A4 harboring binary vector pCAMBIA2301VrNHX1 plasmid on MS basal medium + ascorbic acid (75 mg/L) + kanamycin (150 mg/L) + cefotaxime (400 mg/L).

Experiment	No. of explants inoculated	No. of explants showing resistant roots	Gus^+ analysed roots (%)	PCR^+ (%)
1	15	9	7/9 (77.77)	3/15 (20.00)
2	20	12	4/12 (33.33)	2/20 (10.00)
3	25	13	6/13 (46.15)	3/25 (12.00)
Total	60	34	17	8

Figure 8.1 Schematic construction of salt resistance gene VrNHX1 mobilized with *Agrobacterium rhizogenes* strain A4 harboring the binary vector pCAMBIA2301 containing *npt*II (neomycin phosphotransferase) as selectable marker and the β-glucuronidase (GUS) gene (uidA) with an intron as the reporter gene within the T-DNA region driven by CaMV 35S promoter. LB and RB: left border and right border of T-DNA region.

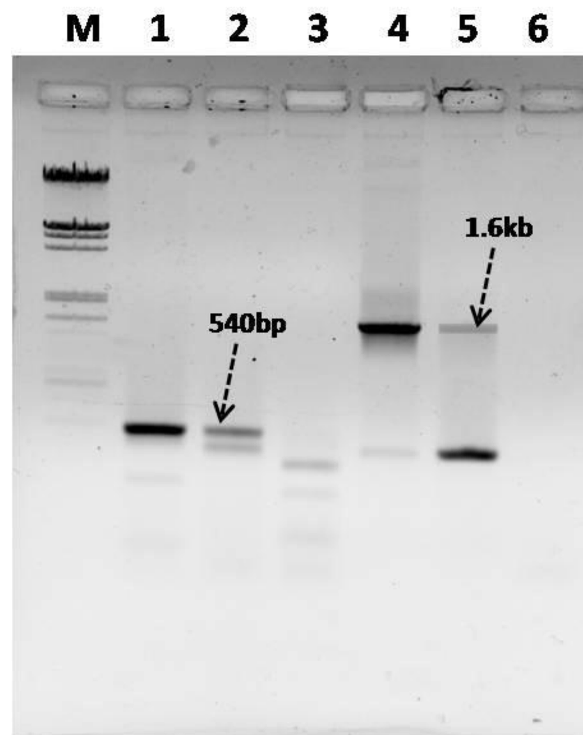

Figure 8.2 Confirmation of the presence of *npt*II and VrNHX1 gene in the T-DNA of the plasmid construct 35SpCAMBIA2301VrNHX1. M-Marker, 1- *npt*II positive control, 2- Sample, 3- *npt*II negative control, 4- VrNHX1 positive control, 5- Sample, 6- VrNHX1 negative control.

Figure 8.3 Histochemical GUS assay on roots of *M. acuminata* cv. Vaibalhla (AAA) in 30 minutes inoculated experiment: Putatively transformed root showing GUS expression.

Figure 8.4 Histochemical GUS assay on roots of *M. acuminata* cv. Vaibalhla (AAA) co-cultivated for 2 day:. **a** – Putatively transformed root showing GUS expression; **b** – Control

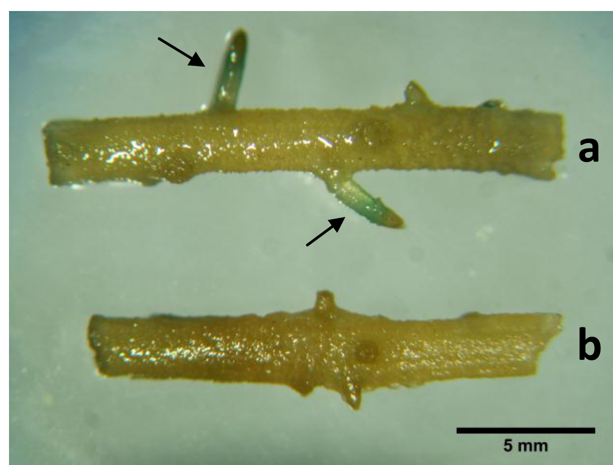

Figure 8.5 Histochemical GUS assay on roots of *M. acuminata* cv. Vaibalhla (AAA) inoculated for 30 minutes and co-cultivated for 2 days: **a** – Putatively transformed root showing GUS expression; **B** – Control

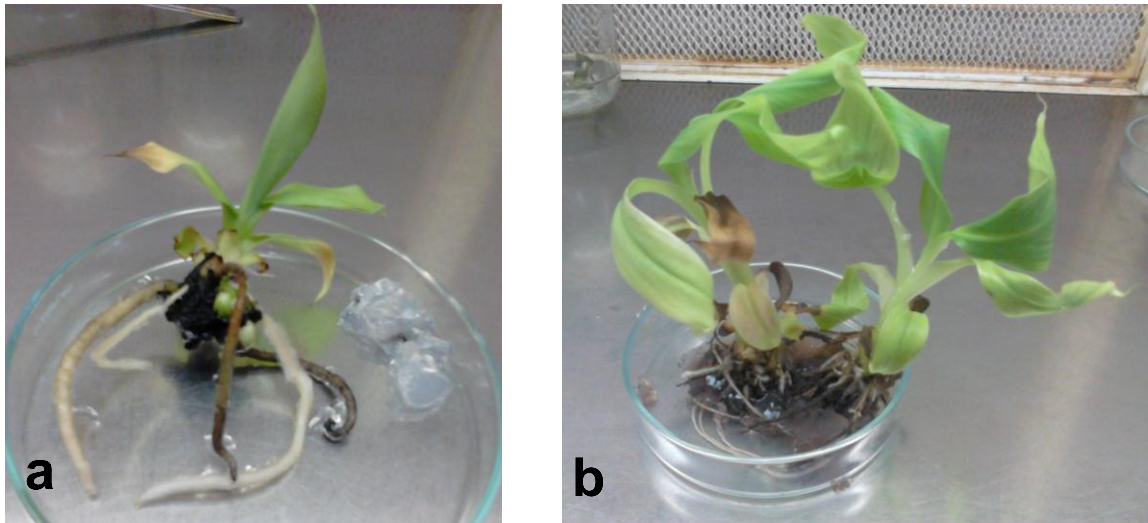

Figure 8.6 Hairy roots of *M. acuminata* cv. Vaibalhla (AAA): **a** – Control plants showing normal roots after 10 days of culture; **b** –Treated plants cultured with *Agrobacterium rhizogenes* strain A4 harbouring binary vector pCAMBIA2301VrNHX1 plasmid on MS basal medium + ascorbic acid (75 mg/L) + kanamycin (150 mg/L) + cefotaxime (400 mg/L) showing hairy roots after 10 days of culture.

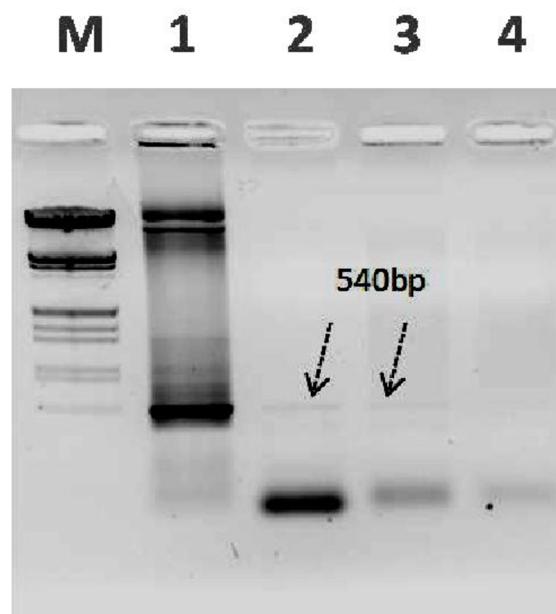

Figure 8.7 Molecular analysis of putatively transformed roots of *M. acuminata* cv. Vaibalhla (AAA): PCR amplification of the 540 bp fragment of the *npt*II gene. M-marker, lane 1- *npt*II positive control, lane 2,3- putatively transformed roots, lane 3- *npt*II negative control.

Summary

SUMMARY

The major findings of the present study are summarized as follows:

- ✓ Three economically important banana cultivars of Mizoram viz. Vaibalhla (*M. acuminata* AAA group), Banria (*Musa* sp. ABB group) and Lawngbalhla (*Musa* sp. AAB group) were identified on the basis of their agronomic and economic potential. Among the three cultivars Vaibalhla was found to be the most popular and fetched the highest price throughout Mizoram.

- ✓ Among the three phytogeographical regions of Mizoram viz. tropical, sub-tropical and temperate regions, the sub-tropical region was found to be the most suitable for the cultivation of the three cultivars.

- ✓ Evaluation of genetic variations within the population of the same cultivar collected from the sub-tropical region (Khumtung, Serchhip district) revealed a close genetic relatedness among the samples studied. However considerable genetic variations were observed among the three cultivars.

- ✓ An efficient *in vitro* regeneration protocol was established for the cultivar Vaibalhla using immature male flowers and suckers as explants. MS medium supplemented with BAP (2 mg/L) and NAA (0.5 mg/L) gave the highest number of white bud-like structures (WBLS) from the male flower explants. Highest percentages of shoot development of the buds were achieved with MS medium supplemented with kinetin (2 mg/L) and NAA (0.5 mg/L). A protocol for the production of synthetic seeds of buds derived from immature male flowers was also established.

- ✓ *In vitro* cultured sucker explants showed the highest response for shoot and root regeneration in MS medium supplemented with BAP (8 mg/L), NAA (2 mg/L) and AdS (50 mg/L). Buds formed from sucker explants within 2-3 weeks were divided into two batches – splitted and un-splitted which were further cultured for more bud formation and regeneration. Splitted explants resulted in more number of shoot buds per explant as compared to the un-splitted buds.

- ✓ An efficient *in vitro* regeneration protocol was also established from the immature male flower explants of an introduced banana *M. acuminata* cv. Grand Naine (AAA) using low concentration of a single plant growth regulator (2 mg/L BAP).

- ✓ The result of the genetic fidelity test conducted for the plantlets derived from immature male flowers of Vaibalhla and Grand Naine with the mother plants using RAPD and ISSR molecular markers showed no detectable genetic variations.

- ✓ *Agrobacterium*-mediated genetic transformation was performed using *Agrobacterium tumefaciens* strain EHA105 harboring pCAMBIA2301AtNHX1 plasmid. The efficiency of immature male flowers and suckers as target explants were analyzed. However the inoculated sucker explants failed to regenerate due to *Agrobacterium* overgrowth. Several attempts have been made to control the overgrowth without any positive response.

- ✓ For the transformation efficiency assay, the un- and pre-cultured immature male flower explants were subjected to various methods of injury such as hypodermal needle injury, with and without sonication and vacuum infiltration. The putative transformation was analyzed using histochemical GUS assay. 14 and 21 days pre-

cultured male flower explants subjected to 4-5 needle point injury resulted in 93.33% and100% putative transformation respectively. 30 s sonication combined with 5 minutes ofvacuum infiltration for 7, 14 and 21 days pre-cultured immature male flower explants gave 73.33%, 66.66% and 71.42% putative transformation respectively.

- ✓ *Agrobacterium*-mediated genetic transformation experiment was also conducted using *Agrobacterium rhizogenes* strain A4 harboring the binary vector pCAMBIA2301VrNHX1 plasmid. The VrNHX1 gene was successfully mobilized with A4 strain using Freeze-thaw method.

- ✓ *In vitro* raised plantlets of *M. acuminata* cv. Vaibalhla (AAA) were targeted for hairy root formation. The plantlets were inoculated with *Agrobacterium rhizogenes* strain A4 harboring pCAMBIA2301VrNHX1for 30 min followed by co-cultivation in dark in MS basal medium for 2 days. The treated plants were transferred and maintained in MS basal medium supplemented with ascorbic acid (75 mg/L), kanamycin (150 mg/L) and cefotaxime (400 mg/L). Gus positive hairy roots initiated within 2 days. The gus positive roots were examined for transient transformation by PCR using *nptII* and NHX1 gene specific primers. Positive bands at 540bp and 1.6 kb confirmed the transfer of the plasmid as well as the gene respectively.

Conclusion

CONCLUSION

Based on the present study 3 commercially important banana cultivars native to Mizoram was identified. The most suitable phytogeographical region was also identified as the sub-tropical region in Mizoram. Successful *in vitro* mass propagation protocols for the production of clonal plantlets, synthetic seed production and *Agrobacterium*-mediated genetic transformation was also conducted.

An efficient method for *in vitro* plant regeneration of *M. acuminata* cv. Vaibalhla (AAA), the most economically important banana cultivar of Mizoram was developed for the first time. Clonally identical plantlets were regenerated from the immature male flowers obtained from the male bud as well as the suckers. The method developed from immature male flowers is efficient and cost-effective, where the male buds of banana were made useful in generating plantlets, to meet the demand for large number of clonal elite suckers. Using suckers as explants, the efficiency of regeneration from splitted and unsplitted explants were studied and compared, where splitted explants were found to give more shoot buds compared to the un-splitted ones. Furthermore, a protocol for developing synthetic seeds from the *in vitro* raised buds derived from male flowers was also established using sodium-alginate beads. The established protocols will be applicable for future conservation and genetic transformation studies of this commercially important banana.

The present study also describes an efficient method for *in vitro* plant regeneration of *M. acuminata* Colla var. Grand Naine (AAA), an economically important Cavendish banana variety in Mizoram, India using low concentration of BAP (2 mg/L). Clonally identical plantlets were regenerated from the immature male flowers obtained from

the male bud. Thus this protocol can also be further scale-up to produce large scale clonal quality planting materials, which will benefit the growers.

The *Agrobacterium*-mediated genetic transformation studies conducted also generated useful information for further studies in the genetic improvement programs.

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Annexures

ANNEXURE (i)

PAPER PRESENTATION/PARTICIPATION IN CONFERENCE/SYMPOSIUM:

1. Participated in the National Conference on "Recent trends on Medicinal Plants Research" held at Centre for Advanced Studies in Botany, University of Madras, Guindy Campus, Chennai from 24th-25th January, 2007.

2. Presented paper in the National Conference on "Natural Resources Management" organized by the School of Earth Sciences and Natural Resources Management, Mizoram University, Aizawl held during 24th and 25th March, 2009.

3. Participated in a DBT sponsored training course on "Bioinformatics - General Concepts and Applications" organized by the Bioinformatics Infrastructure Facility, Department of Biotechnology, Mizoram University during March 26-27, 2009.

4. Attended a DBT Program Support Project sponsored Short-term course on "Application of Molecular Tools for Crop Improvement" held at Indian Institute of Technology Guwahati organized by the Department of Biotechnology from 16th-21st November, 2009.

5. Participated in a DBT sponsored "Entrepreneurship Development Programme in Biotechnology" organized by Biotech Consortium India Limited (BCIL), New Delhi and Mizoram Council of Science, Technology and Environment, Aizawl held at Aizawl, Mizoram from 19th -22nd January, 2010.

6. Presented paper the National Conference on "Conservation of Biodiversity" organized by the National Centre for Science Communicators.held at Indian Institute of Science, Bangalore, 560012 from 20th and 21st November, 2010

7. Presented poster in the National Symposium on "Recent advances in Plant Tissue Culture and Biotechnological Researches in India" and XXXII Annual

Meet of Plant Tissue Culture Association (India) organized by the M.N. Institute of Applied Sciences, Bikaner (Rajasthan) held from 4th-6th February 2011.

8. Participated in the DBT sponsored workshop on "Bioinformatics - Structure and Determination of Macromolecules" organized by the Department of Biotechnology, Mizoram University during March 28-29, 2011.

9. Participated in the DBT sponsored training course on "Bioinformatics - Proteins and their structure prediction" organized by the Department of Biotechnology, Mizoram University during November 23-24, 2011.

10. Presented paper in the National Seminar on "Emerging Trends in Biosciences and Future Prospects" organized by the Department of Zoology, Pachhunga University College, Aizawl, Mizoram in collaboration with the Department of Zoology, Mizoram University, Aizawl, Mizoram held on 29th- 30th November 2011.

11. Participated in the Indo-Japan DBT Workshop for "Fostering Research, Collaboration and Innovation in Translational Bioresources in Northeast India", organized by the DBT Program Support Center at Department of Biotechnology, Indian Institute of Technology Guwahati on 1st October 2012.

12. Participated as a member of organizing committee in the "5th Interactive Meeting of North East Bioinformatics Centres Network (NEBInet)" organized by the Department of Biotechnology, Mizoram University, Aizawl - 796004 from 11th-12th October, 2012.

13. Participated in India-UK Scientific Seminar on 'Structural Elucidation of Microbial Natural Products: Opportunities and Challenges' organized by the Department of Biotechnology, Mizoram University, India jointly funded by Department of Science and Technology (DST), Govt. of India, New Delhi and The Royal Society, London, U.K. from 15th -17th January, 2014.

14. Participated in the "One day seminar on genetically modified crops and food security" held on 23rd January 2014 organized by Mizo Academy of Sciences at Government Zirtiri Residential Science College, Aizawl, supported by Directorate of Science and Technology, Government of Mizoram, Catalysed and supported by the National Council for Science and Technology Communication, Department of Science and Technology.

ANNEXURE (ii)

LIST OF PUBLICATIONS

Abstract:

1. **L.Hrahsel,** S.Saravanan and R.Thangjam (2009) *In vitro* regeneration studies of *Tectona Grandis* L. In: Proceedings of the 96th Indian Science Congress organized at North-Eastern Hill University, Shillong. January 3-7.

2. **Lalremsiami Hrahsel** and Robert Thangjam (2009) Prospects of Plant Tissue Culture Techniques for Sustainable Production of Banana in Mizoram. In: National Conference on Natural Resources Management in Mizoram University, Aizawl, Organized by School of Earth Sciences and Natural Resources Management, Mizoram University during 24-25 March.

3. **Lalremsiami Hrahsel** and Robert Thangjam (2010) Application of Tissue Culture Techniques for Sustainable Utilization of Banana in Mizoram. In: Proceedings of the National Conference on "Conservation of Biodiversity" the National Centre for Science Communicators, held at Indian Institute of Science, Bangalore, 560012.

4. **Lalremsiami Hrahsel** and Robert Thangjam (2011) *In vitro* mass propagation of an introduced Cavendish banana (*Musa acuminata* Colla) var. Grand Naine in Mizoram, India. In: Proceedings of the national seminar on emerging trends in biosciences and future prospects organized by Department of Zoology, Pachhunga University College, Mizoram University, Aizawl, 29-30 November. Published in Science Vision, 11(4) (ISSN 0975-6175).

5. **Lalremsiami Hrahsel** and Robert Thangjam (2011) *In vitro* regeneration of *Vanda coerulea* Giff. via protocorm-like body (PLB) formation. In: National Symposium on Recent Advances in Plant Tissue Culture and Biotechnological Researches in India & XXXII Annual Meet of Plant Tissue Culture Association (India) organized by M.N. Institute of Applied Sciences, Bikaner (Rajasthan), 4-6th February.

Full length papers:

1. Robert Thangjam, **Lalremsiami Hrahsel** and P.C. Lalrinfela (2009) Prospects of Biotechnological Intervention for Sustainable Utilization of Banana Genetic Resources in Mizoram, India. In: Newsletter of North East India Research Forum (Ningthoujam D, Nath T, Sharma M, Laishram *et al*. eds.). N.E. Quest, 3 (1): 39-44.

2. **Lalremsiami Hrahsel** and Robert Thangjam (2013) Strategies for Large Scale Production of Commercially Important Banana Varieties. Science Vision, 13 (3): 137-141.

3. **Lalremsiami Hrahsel,** Adreeja Basu, Robert Thangjam and Lingaraj Sahoo (2013) *In vitro* propagation and assessment of genetic fidelity of *Musa acuminata* (AAA) cv. Vaibalhla derived from immature male flowers. Applied Biochemistry and Biotechnology, 172:1530–1539 doi: 10.1007/s12010-013-0637-9. IMPACT FACTOR:1.893 (2012)

4. Thangjam Premabati, **Lalremsiami Hrahsel**, Pachuau Lalrinfela and Robert Thangjam (2013) Evaluation of genetic diversity among edible banana varieties found in Mizoram, using randomly amplified polymorphic DNA. Journal of Plant Breeding and Genetics, 01 (03): 149-155.

5. H. Lallawmawma, P.C. Lalrinfela, **Lalremsiami Hrahsel**, Atom Annupama Devi, Thangjam Premabati and Robert Thangjam (2013) DNA barcoding as a tool for rapid identification of plants: a case study in banana family (Musaceae) In: Bioresources and traditional knowledge of Northeast India (Singh KK, Das KC and Lalruatsanga H eds.). Mizo Post Graduate Science Society (MIPOGRASS), Aizawl, India, pp. 21-34.

Prospects of Biotechnological Interventions for Sustainable Utilization of Banana Genetic Resources in Mizoram, India

By Robert Thangjam, Lalremsiami Hrahsel and P. C .Lalrinfela*

Abstract

Mizoram lies within the north eastern region of India considered as the centre of diversity of banana. 20 clones across 3 species have been documented using morphological data. However, there is a need for proper characterization of these clones using molecular tools in order to understand their genetic make up and relationships. With the loss of crop genetic resources at an alarming rate the future of global food crops depends on the sustainability of the genetic pool at their centres of diversity. Biotechnological approaches through the application of tissue culture techniques provide one of the most reliable and time tested options for the sustainable production of banana. This article discusses some of the molecular tools that can be applied for better understanding of banana genetic resources in Mizoram.

Introduction

Banana is a major fruit crop growing in more than 120 countries with India as the top producer in the world. It is greatly diversified in North-East India including the state of Mizoram. Banana belongs to the family *Musaceae*, which consists of two genera: *Musa* and *Ensete*. Genus *Ensete* has 9 species while *Musa* genus has four sections namely, *Eumusa, Rhodochlamys, Australimusa* and *Callimusa* (Simmonds and Shepherd, 1955). The Eumusa constitutes the source of edible bananas chiefly belonging to *M. acuminata* and *M. balbisiana*. *M. acuminata* has been divided into 8 sub-species whereas *M. balbisiana* is less morphologically diversified (Roux *et al.,* 2008). India is the largest producer of banana with annual production of 13.5 mt from an area of 4.0 lakh ha (Daniells *et al.,* 2001). Besides the cultivated species and their cultivars, majority of the species of *Musa* were found in wild conditions and they are widely distributed in the northeastern states.

Banana status in Mizoram:

The north-eastern region of India have been considered as the richest sources of natural banana diversity where *M. balbisiana* from Indian subcontinent meet *M. acuminata* from South East Asia (Molina and Kudagamage. 2002). However, there are very few reports on the genetic resources of wild and edible bananas from Northeast India including Mizoram. Hore *et al.,* (1992) reported 4 species under the genus *Ensete* and 10 species under *Musa.* Uma and Sathiamoorthy (2002) characterize 20 different bananas and plantains of Mizoram including their tentative genome groups using morphological characters.

Mizoram is endowed with three different climatic condition such as tropical, sub-tropical and temperate zones. *Changthir* and *Banria* are the predominant cultivars distributed throughout Mizoram. *Amrit sagar*, a unique AAA cultivar is popular among small-scale and backyard growers. This variety is popularly known as *Vaibalhla* or *Cavendish*. Banana is referred as *Balhla* in Mizo language like Kola, Kela or Vazhai. In the northern provinces, *Cheeni champa* is commercially grown in areas of Bhaga Bazar, Vairengte, Bilkhawthlir, Kolasib,etc. Apart from many cultivars, many wild types are also abundantly grown. These are preferred for their male buds to be used as vegetables. Of all the wild types, *Sai su* (*Ensete glaucam*) is the most unique wild type.

Need for biotechnological approaches

1. Characterisation of banana genetic resources in Mizoram

Earlier investigations and collections of the germplasms reported from Mizoram were not representative of the total banana genetic resources of Mizoram as the sites and samples studied were very small. Moreover, the identification and characterization of germplasms is heavily influenced by environmental factors thereby limiting their uses. Thus the use of molecular tools is essential to validate the genetic status. Nair *et al.,* (2005) classified banana cultivars into two genomic groups by scoring morphological features. Earlier attempts in genomic characterization and genetic diversity studies were successfully done with molecular markers such as RAPD (Williams *et al.,* 1990), AFLP and microsatellites (Onguso *et al.,* 2004; Wong *et al.,* 2002; Creste *et al.,* 2004) Molecular markers provided a quick and reliable method for genomic characterization.

2. Analysis of resistance gene in the banana gene pool of Mizoram

The banana cultivars are originated from the intra and inter – specific hybridisation of two wild diploid species *M. acuminata* and *M. balbisiana.* The different ploidy status, progenitor species, sterility and interspecific compatibility has led to various genomic compositions. Most of the present edible bananas are triploid, a few cultivars are diploids and tetraploids. These cultuvars normally lack sources of resistance to pests and diseases The majority of the cloned disease resistance genes (R-genes) in plant species encode a large family of the nucleotide-binding site/leucine-rich repeat (NBS-LRR) proteins, which are characterized by various domains including a variable N-terminal domain, a nucleotide-binding site and a C-terminal LRR motif. PCR amplification with degenerate primers targeting to short conserved region in NBS is an efficient method for identifying resistance gene analogues (RGAs). This method has been successfully used for isolation of NBS-LRR gene from a wide variety of plant species (Leister *et al.,* 1996; Xiao *et al.,* 2006). Cultivated bananas, which were originated from natural intra- and inter-specific hybridization of *M. acuminata* and *M. balbisiana*, are highly susceptible to various viral and fungal diseases. The genetics and diversity of the resistance genes (R-genes) are poorly understood. For future genetic improvement and breeding purposes, a clear knowledge of the diversity and phylogenetic relationship of the genetic make-up in the wild and cultivated bananas is highly essential.

3. Production of quality planting materials in Mizoram

Banana is a long duration crop of one and a half years and is propagated vegetatively by suckers. The production of suckers varies in different genotypes ranging from 5-10 per plant per year. Crop productivity and maturity is dependent on the size and age of suckers and uneven maturity extends the duration by 3-4 months. Suckers also carry soil nematodes, disease causing organisms such as bunchy top virus, leaf spot etc., thereby affecting the crop production considerably. In this regard, biotechnological approaches such as cell and tissue culture, protoplast fusion and gene transfer may serve as useful tools (Novak *et al.,* 1993; Ganapathi *et al.,* 2002). *In vitro* propagation of banana through shoot tip cultures is useful in the rapid multiplication of desirable disease free plantlets. In addition, careful selection and updating of

mother plants result in improved crop yield (Vuylsteke, 1989).
For the large-scale sustainable production of banana, a large number of superior quality planting materials is required, which is difficult to obtain by conventional methods of propagation. In contrast, micropropagation through tissue culture techniques offers rapid and reliable means of producing large number of genetically uniform clonal planting material within a short time. Despite the availability of many reports on *in vitro* propagation in bananas, in which the protocols are complicated, the standardization of specific protocols for a specific cultivar is essential. Development of new banana varieties through conventional breeding programs remains difficult because of sterility and polyploidy of most edible cultivars.
There is a further need to develop somatic embryogenesis techniques for the mass propagation of desirable clones. The scale-up and automation of techniques necessary to reduce the costs of production further should be investigated. In addition, field-testing of plants regenerated from cell culture should be investigated.

Strategies for biotechnological approaches

1. Molecular characterization of banana genetic resources in Mizoram
i) Survey and collection of wild and cultivated banana plants growing in different phytogeographical regions of Mizoram:
Proper and exhaustive survey and collection for the wild and cultivated banana plants should be conducted in different regions of Mizoram. Maximum areas under different phytogeographical regions should be covered in the germplasm collection. The germplasms should be maintained in the field gene bank giving different accession numbers and a duplicate will be submitted to NBPGR for validation.
ii) Identification and characterization of collected banana plants based on their morphological scores and molecular tools:
a) Morphological characterization: For identification of the collected germplams, the classification of Simmonds and Shepherd (1955) should be used and using IPGRI descriptors (1996), the genomes of the germplasms should be classified.
b) Molecular Characterization: For validation of the genome groups, molecular tools such as IRAP (Inter-Retrotransposons Amplified Polymorphism) markers (Nair *et al.*, 2005) can be used. The accessions belonging to the same genome group can be characterized for their genetic variation using RAPD (Williams *et al.*, 1990). The resistance genes with nucleotide-binding site/leucine-rich repeat proteins (NBS-LRR) can be isolated from selected representatives of the wild and edible bananas using PCR amplification of the genomic DNAs using degenerate primers. The PCR products with the desired fragments will be purified, cloned and sequenced. The sequences can then be aligned using BLAST programs and analysed for its phylogenetic relationship.

2. Sustainable production and utilization of banana in Mizoram
i) **Characterization of the different cultivars** of edible banana grown in different phytogeographical regions of Mizoram and identification of the superior genotypes:
Popular local banana cultivars viz., *vai balhla kual*, *lawng balhla*, *banria*, etc., that are of economic importance need to be collected from different phytogeographical regions of Mizoram and the prospective mother plants be thoroughly evaluated and selected for its superior agronomic characteristics

and disease resistance. For the validation of superior genotypes, molecular tools such as RAPD (Williams *et al.,* 1990) can be used.

ii) **Standardization of *in vitro* regeneration systems of superior genotypes** for rapid multiplication of genetically stable planting materials.

a) **Initiation of aseptic culture**: Various explants (male flower buds, shoot tips, immature zygotic embryos,) taken from the selected superior mother plants materials can tested for their response in various culture media such as MS (Murashige and Skoog, 1962), B5 (Gamborg *et al.,* 1968), White media (White, 1943), etc. under standard culture conditions. The effect of various growth regulators such as cytokinins, auxins, etc. on the *in vitro* regeneration potential of selected banana genotypes should be evaluated.

b) ***In vitro* regeneration**: Some of the explants from the above culture may result in direct or indirect regeneration of shoots or roots. The obtained *in vitro* organs can be transferred to different media and hormonal combinations for further multiplication and rooting.

c) **Hardening and acclimatization**: The rooted regenerated plantlets should be transferred into pots containing sand and soil mixture for primary hardening in a growth chamber, and then proceeding for secondary hardening and acclimatization in the polyhouse under standard conditions. The successfully hardened and acclimatized plantlets should then be transferred to the field.

iii) **Standardization of in vitro regeneration systems from encapsulated aseptic cultures**: The established aseptic cultures such as flower buds, shoot buds, somatic embryos can be encapsulated with the help of sodium alginate and regenerated into plantlets.

iv) **Genetic fidelity testing of the regenerated plantlets**: For testing of genetic fidelity of the regenerated plantlets, leaf samples of the hardened plantlets should be used for isolation of genomic DNA and compared with mother plants using RAPD or SSR markers.

Conclusion

With the application of biotechnological tools, proper understanding and knowledge of the status, genome classification and genetic resources of the wild and cultivated banana plants growing in Mizoram can be achieved. Further, identification and characterization of the resistance genes (R-genes) and understanding of their phylogenetic relationship among the different species and/or cultivars of bananas grown in Mizoram can also be carried out for future genetic improvement programs. Since Mizoram is located in the centre of diversity of *Musa* germplasm, it is imperative to take necessary steps at all levels for conservation and sustainable production of banana genetic resources. Application of biotechnological tools such as *tissue culture* and *DNA profiling* techniques could serve as the best option for these programs.

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Banana, raw, edible parts
Nutritional value per 100 g (3.5 oz)

Energy 90 kcal 370 kJ

Carbohydrates 22.84 g

- Sugars 12.23 g

- Dietary fiber 2.6 g

Fat 0.33 g

Protein 1.09 g

Vitamin A equiv. 3 μg 0%

Thiamine (Vit. B1) 0.031 mg = 2%

Riboflavin (Vit. B2) 0.073 mg = 5%

Niacin (Vit. B3) 0.665 mg = 4%

Pantothenic acid (B5) 0.334 mg = 7%

Vitamin B6 0.367 mg = 28%

Folate (Vit. B9) 20 μg = 5%

Vitamin C 8.7 mg = 15%

Calcium 5 mg = 1%

Iron 0.26 mg = 2%

Magnesium 27 mg = 7%

Phosphorus 22 m g= 3%

Potassium 358 mg = 8%

Zinc 0.15 mg = 1%

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Strategies for large-scale production of commercially important banana varieties of Mizoram, India, using plant tissue culture technique

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Abstract

The technique of plant tissue culture has been well accepted and applied in the mass propagation of planting materials in various crops and plants. In India numerous micropropagation units are producing millions of plantlets catering the needs for the increasing demand of quality planting materials. The advantages of this technique lie in the production of plantlets that are disease free and genetically identical to the elite mother plants. Application of plant tissue culture technique is the only viable means for the large scale production of banana planting materials which is not possible through conventional propagation. The article discusses the strategies of the mass production of commercially important banana in Mizoram using plant tissue culture techniques.

Key words: Banan; micropropagation; tissue culture; Mizoram.

Introduction

Plant tissue culture is the science of growing plant cells, tissues or organs isolated from the mother plant, on artificial media under aseptic conditions. The plant tissue culture technology owes its origin to the concept of totipotency of cell, introduced by Haberlandt, 1902 which led to the successful culture of tomato roots.[1] The number of successful cases is continuously increasing. Numerous publications and reports have come out with regard to the basic procedures and method involved in plant tissue culture in various plants.[2-4] The simpler techniques that are found to be applicable directly in propagation and genetic improvement of plants are (i) micropropagation, (ii) meristem culture, (iii) somatic embryogenesis, (iv) somaclonal variation, (v) embryo culture, (vi) *in vitro* selection, (vii) anther culture, and (viii) protoplast culture.[5] For the large-scale sustainable production of plants, a number of superior quality planting materials is required, which is difficult to obtain by conventional methods of propagation. In contrast, thousands of plants could be derived from a single cell or tissue in a relatively short amount of time, thus having a great potential for

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mass propagation of commercially important crops such as banana. Initial plant material could be obtained from an inflorescence, proliferating meristem, zygotic embryos and rhizome and leaf sheaths; depending on the type of species at hand. This technology could thus be commercially used for mass propagation of quality planting materials by micropropagation (i.e. *in vitro* clonal propagation), mass production of quality and useful secondary metabolites, development of new varieties (via mutagenesis, etc.) as well as the production and enhancement of natural pharmaceutical, nutraceutical and cosmaceutical compounds. Plant cell culture is a requirement as well for genetic engineering in the production of designer plants, whereby these could be designed for desirable attributes such as disease resistance, tolerance towards environmental strains and mass production of antibodies and bioplastics. It can become a versatile tool for mass multiplication of elite clones, elimination of disease in planting material, creation of super genotypes of agricultural crops, which hither to it was not possible through conventional plant breeding methods. Tissue culture propagation can thus heighten our ability to produce consistently uniform superior planting material for export and domestic market. Micropropagation through tissue culture techniques thus offers rapid and reliable means of producing large number of genetically uniform clonal planting material within a short time.

Advantages of Plant Tissue Culture Technique

- ⊕ Micropropagation results in rapid propagation of a superior plant while maintaining the genetic make-up and also helps in storage of germplasm.
- ⊕ Established aseptic cultures such as flower buds, shoot buds, somatic embryos may be packaged as artificial seeds which are encapsulated for distribution and protected with a complex of agar and other gel-forming compounds such as sodium-alginate beads, and stored in a protective, hydrated gel with nutrients for a long period under ultra low temperature.[6] Slow growth techniques can also be applied for the maintenance of the culture for a longer time in limited culture media.
- ⊕ The plantlets that are derived from these techniques are free from fungal and bacterial diseases since the contaminated plants fail to respond and gradually die out. Viral diseases could be eliminated from plant propagative material through quarantine and virus indexing. These checks are recommended for verification of the disease-free planting materials.[7]
- ⊕ Somaclonal variations derived from callus and cell suspension cultures can be utilized for the induction of desirable, heritable changes in regenerated plants by subjecting a population of cells to a selection pressure.
- ⊕ Regeneration from callus, cell suspention and pollen cultures helps to produce homozygous, pure-breeding lines of plants for hybrid production and genetic studies and also to improve the efficiency of *in vitro* selection. The use of colchicine may be needed to double the chromosome number of haploid plants.[8-9]
- ⊕ Protoplast culture helps to incorporate potentially useful genes from one plant species to another by fusion of protoplast and regeneration from the hybrid cell line. It also helps to transfer specific genes into protoplasts and regenerate transgenic plants.[10]
- ⊕ Plant cell and tissue culture can also be used for large scale harvesting of medicinally important secondary metabolites, which otherwise, will need a large number of plants from the natural population. Similarly, there are number of cultured cells producing metabolites not synthesized by the plant itself e.g. *Lithospermum erythrorhizon* cultures have been observed to synthesize rosmarinic acid - a characteristic of lemon plants, through metabolic engineering.[11]

Application of Plant Tissue Culture Technique for Production of Quality Planting Material in Banana

In spite of the availability of many reports on *in vitro* propagation in banana, in which the protocols are complicated, the standardization of specific protocols for a specific cultivar is essential. Development of new banana varieties through conventional breeding programs remains difficult because of sterility and polyploidy of most edible cultivars. Banana being one of the most widely distributed fruit crops in the world, it is cultivated in more than 120 countries covering about 10 million hectares, with an annual production of 130 million tons.[12] It is the fourth most important food crop after rice, wheat and maize.[13] The crop is strongly believed to have originated from Southeast Asia, and many of the species and clones have India as their homeland.[14] Natural hybridization, mutation and polyploidy have contributed a lot for wide diversity among Indian bananas which have perpetuated through vegetative propagation over.

As a result of various shortcomings including lack of uniformity, high disease and pest infection rates, as well as the bulkiness of conventional propagation via suckers, the application of various biotechnological approaches has become an integral part of the banana industry.[15] In particular, the use of plant tissue culture via clonal propagation of superior cultivars has been an immense benefit to commercial banana farmers globally.[16] *In vitro* propagation provides excellent advantages over traditional propagation, including a high multiplication rate, physiological uniformity, the availability of disease-free material all the year round, rapid dissemination of new plant materials throughout the world, uniformity of shoots, short harvest interval in comparison with conventional plants, and faster growth in the early growing stages compared to conventional materials.[17-19]

Banana is a long duration crop of one and a half years. The production of suckers varies in different genotypes ranging from 5-10 per plant per year. Crop productivity and maturity is dependent on the size and age of suckers and uneven maturity extends the duration by 3-4 months. Suckers also carry soil nematodes, disease causing organisms such as bunchy top virus, leaf spot etc. thereby affecting the crop production considerably. In this regard, biotechnological approaches such as cell and tissue culture, protoplast fusion and gene transfer offer as useful tools.[20] *In vitro* propagation of banana through shoot tip cultures is useful in the rapid multiplication of desirable disease free plantlets. In addition, careful selection and updating of mother plants results in improved crop yield.

Strategies for Production of Quality Planting Material of Banana in Mizoram

North-east India is considered as the reservoir for the large gene pool of banana genetic resources, and is the meeting point of *Musa balbisiana* of the Indian subcontinent and *Musa acuminata* of Southeast Asia.[21] With the loss of crop genetic resources at an alarming rate, the future of global food crops depend on the sustainability of the genetic pool at their centre of diversity. The northeastern states of India, namely Assam, Arunachal Pradesh, Meghalaya, Tripura, Mizoram and Manipur have been richest sources of natural diversity. Altogether 39 different accessions of banana have been collected and characterised.[14] From the state of Mizoram, 14 different accessions have been collected and characterized.[22] The important commercial banana varieties of the state are vaibalhla (*M. acuminata* AAA group), lawng balhla (*Musa* AAB group) and banria (*Musa* ABB group). The strategies for mass propagation of these commercially important banana varieties are as follows:

1. Characterization of the different cultivars of edible banana grown in different phytogeographical regions of Mizoram and identification of the superior genotypes:

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The prospective mother plants should be thoroughly evaluated and selected for its superior agronomic characteristics and freedom from diseases. The selected plants should be maintained in a protective area where soil and plantation hygiene are in place. For the proper validation of superior genotypes, molecular markers such as random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), simple sequence repeat (SSR) can be used. Continuous evaluation for disease signs and symptoms should be made in the plantation areas and the plants itself. Viral diseases like banana bunchy top virus (BBTV) should be screened using enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) techniques. The plantation area should be free from any disease plants. Only the plants that are healthy looking, having superior agronomic traits and free from viral symptoms should be selected as mother plants for the mass propagation.

2. Standardization of in vitro regeneration system of superior genotypes for rapid multiplication of genetically stable planting materials:

a) Initiation of aseptic culture: Various explants (male flower buds, shoot tips, immature zygotic embryos) taken from the selected superior mother plants materials can be tested for their response in various culture media such as MS,[23] B5,[24] White media,[25] etc. under standard culture conditions. The effect of various growth regulators such as cytokinins, auxins, etc. on the *in vitro* regeneration potential of selected banana genotypes should be evaluated.

b) *In vitro* regeneration: Some of the explants from the above culture may result in the direct or indirect regeneration of shoots or roots. The obtained *in vitro* organs can be transferred to different media and hormonal combinations for further multiplication and rooting.

c) Hardening and acclimatization: The rooted regenerated plantlets should be transferred into pots containing sand and soil mixture for primary hardening in a growth chamber then proceed for secondary hardening and acclimatization in the polyhouse under standard conditions. The successfully hardened and acclimatized plantlets should be transferred to the field.

3. Standardization of in vitro regeneration system from encapsulated aseptic cultures:

The established aseptic cultures such as flower buds, shoot buds, somatic embryos can be encapsulated with the help of sodium alginate and regenerated into plantlets.

4. Genetic fidelity testing of the regenerated plantlets:

For testing of genetic fidelity of the regenerated plantlets, leaf samples of the hardened plantlets should be used for isolation of genomic DNA and compared with mother plants using RAPD or SSR markers.

Conclusion

Mizoram is located in the centre of diversity of Musa germplasm which indicates that it is imperative to take necessary steps at all levels for the conservation and sustainable production of banana genetic resources. Application of biotechnological tools such as tissue culture and DNA profiling techniques could serve as the best option for these programs.

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In Vitro Propagation and Assessment of the Genetic Fidelity of *Musa acuminata* (AAA) cv. Vaibalhla Derived from Immature Male Flowers

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Abstract An efficient in vitro propagation method has been developed for the first time for *Musa acuminata* (AAA) cv. Vaibalhla, an economically important banana cultivar of Mizoram, India. Immature male flowers were used as explants. Murashige and Skoog's (MS) medium supplemented with plant growth regulators (PGRs) were used for the regeneration process. Out of different PGR combinations, MS medium supplemented with 2 mg L^{-1} 6-benzylaminopurine (BAP) + 0.5 mg L^{-1} α-naphthalene acetic acid (NAA) was optimal for production of white bud-like structures (WBLS). On this medium, explants produced the highest number of buds per explant (4.30). The highest percentage (77.77) and number (3.51) of shoot formation from each explants was observed in MS medium supplemented with 2 mg L^{-1} kinetin + 0.5 mg L^{-1} NAA. While MS medium supplemented with a combination of 2 mg L^{-1} BAP + 0.5 mg L^{-1} NAA showed the maximum shoot length (14.44 cm). Rooting efficiency of the shoots was highest in the MS basal medium without any PGRs. The plantlets were hardened successfully in the greenhouse with 96 % survival rate. Random amplified polymorphic DNA (RAPD) and inter-simple sequence repeat (ISSR) markers were employed to assess the genetic stability of in vitro regenerated plantlets of *M. acuminata* (AAA) cv. Vaibalhla. Eight RAPD and 8 ISSR primers were successfully used for the analysis from the 40 RAPD and 30 ISSR primers screened initially. The amplified products were monomorphic across all the regenerated plants and were similar to the mother plant. The present standardised protocol will find application in mass production, conservation and genetic transformation studies of this commercially important banana.

Keywords In vitro propagation · Male flower · Musa AAA group · Genetic stability · RAPD · ISSR

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Introduction

Banana, *Musa* spp. (Musaceae), is one of the most widely distributed fruit crops in the world, grown in more than 120 countries covering almost 10 million hectares, with an annual production of 95 million tonnes. It is the fourth most important food crop after rice, wheat and maize [1] and originated from Southeast Asia, where many of the species and clones are native to India [2]. More than 970 indigenous bananas from all over India were collected and maintained in the National Research Centre on Banana (NRCB), Trichy, Tamil Nadu. Out of these, 109 were collected from north eastern states of the country [1].

The northeastern region of India is considered as one of the meeting points of *Musa balbisiana* from Indian subcontinent and *Musa acuminata* from Southeast Asia [3]. Thus, the region has rich natural diversity, and 39 different banana accessions have been collected and characterised [2]. Thirteen commercially important edible cultivars have been identified from this region, out of which, five were from the state of Mizoram [1]. The triploid (AAA) cultivar Vaibalhla is the most widely cultivated and commercially important banana in Mizoram, having a characteristic of sweet taste and texture [4]. However, the large-scale production of this cultivar is hampered by the lack of disease-free quality planting materials.

In recent times, several diseases and pests have affected global banana production largely due to the unavailability of adequate number of disease-free quality planting clones [5–8]. Banana diseases such as bunchy top (bunchy top virus), black Sigatoka (*Mycosphaerella fijiensis*) and yellow Sigatoka (*Mycosphaerella musicola*) are prevalent in northeast India [2]. Improvement by traditional breeding procedure is difficult and time-consuming because of the high sterility, polyploidy and long generation time of most edible varieties. Thus, tissue culture techniques can offer faster and reliable means of producing large number of genetically uniform clonal planting material within a short period of time. Several reports on in vitro propagation in banana are available, but there is still the need for standardisation of specific protocols for economically important cultivars like Vaibalhla. Regeneration via callus phase is less desirable for in vitro plant recovery due to high frequencies of genetic distortions [9–11]. Micropropagation using banana male floral meristems showed no detectable somaclonal variation and found to show less risk for possible virus contamination [12] as compared to soil grown suckers. Moreover, there is an opportunity to select male buds with desirable characteristics such as greater number of hands and fruits per bunch [13], which help to increase the efficiency of micropropagation and produce plantlets from the parts which could be lost during harvesting, when in normal cases, male buds serve only as food or fodder. Thus, the demand for a large number of suckers of elite cultivars can be met using inflorescence tip cultures [14].

The genetic fidelity of regenerated plants of banana is often questioned since there are frequent reports on the occurrence of somaclonal variations not only in regenerated plants but also in micropropagated ones [9–11]. Micropropagation of banana is a major activity in most of the commercial tissue culture units where high levels of growth regulators are often used to enhance the rate of shoot multiplication [15–17]. Similarly, the holding of shoot cultures for long periods in vitro during micropropagation is known to result in undesirable clonal variability in important commercial crops such as *Anigozanthos viridis* [18], *Prunus dulcis* [19], *Foeniculum vulgare* [20], and *Chlorophytum arundinaceum* [21]. Randomly amplified polymorphic DNA (RAPD) [22] and inter-simple sequence repeats (ISSR) [23] markers have proved to be efficient in detecting genetic variations. While there are several reports on genetic analyses of micropropagated banana plants, there are fewer reports on the regenerated ones. The present study reports the successful in vitro regeneration of *M. acuminata* (AAA) cv. Vaibalhla derived from immature male flowers and evaluation of their genetic fidelity using RAPD and ISSR markers.

Materials and Methods

Plant Material

Immature male flowers obtained from the male bud of *M. acuminata* (AAA) cv. Vaibalhla (Fig. 1a) maintained at the field gene bank in the Department of Biotechnology, Mizoram University, Aizawl was used as the explant source. The male buds (Fig. 1b) were shortened to 4–6 cm in length (Fig. 1c) by removing the enveloping bracts, washed with labolene for 30 min, and rinsed with tap water. The explants were brought under laminar air flow chamber where sterilisation was proceeded with 70 % (*v*/*v*) ethanol for 5 min, then with 1 % sodium hypochlorite for 5 min and finally rinsed with sterile distilled water (4–5 times). The sterilised buds were dissected under aseptic conditions, and individual male flowers of size 15–30 mm (Fig. 1d) were kept aside for culture.

Fig. 1 Different stages of the in vitro regeneration of *M. acuminata* (AAA) cv. Vaibalhla derived from immature male flower explants. **a** Mother plant bearing fruits and male bud. **b** Male floral bud. **c** Reduced floral bud (4 cm). **d** Individual immature male flowers dissected separately. **e** Formation of white bud-like structures (WBLS) in MS + BAP (2 mg L^{-1}) + NAA (0.5 mg L^{-1}) after 5 weeks. **f** In vitro shoot clusters. **g** Plantlet ready for hardening. **h** Secondary hardened plant

In Vitro Regeneration

Individual male flowers were transferred to Murashige and Skoog (MS) basal medium [24], supplemented with different concentrations (0, 1.0, 2.0, 3.0, 4.0 and 5.0 mg L^{-1}) of 6-benzylaminopurine (BAP), kinetin, α-naphthalene acetic acid (NAA) or 2,4-D (2,4-Dichlorophenoxyacetic acid) in single and in combinations for initial culture as well as for shoot proliferation and multiplication. The media were solidified with 0.8 % (*w*/*v*) agar and sterilised for 15 min at 121 °C (15 lb psi pressure). Cultures were incubated at 25±1 °C under 16:8 h light and dark photoperiod with light intensity of 55 μmol m^{-2} s^{-1} using cool white fluorescent light (Bajaj Ltd., India) and 50–60 % relative humidity (RH). The morphogenetic changes observed in the culture were recorded weekly. The number of white bud-like structures (WBLS) formed per explant was recorded and subsequently transferred on a shoot proliferation medium containing MS media supplemented with different concentrations of BAP (2, 4 mg L^{-1}), kinetin (2, 5 mg L^{-1}) and NAA (0.5 mg L^{-1}) either in single or in combinations. The number of shoots per bud and the plantlets with fully expanded leaves obtained in shoot proliferation medium were recorded. The evaluation of rooting percentage and numbers per plantlet were recorded by sub-culturing in MS media with and without plant growth regulators (PGRs). Plantlets having fully expanded leaves and well-developed roots were transplanted for primary hardening in the laboratory containing sterilised soil: sand (1:1) and covered with polythene to maintain humidity. They were successfully transferred to greenhouse in pots containing farmyard manure: sand (1:1). The established plants were apparently uniform morphologically and did not show any detectable variation. Fresh leaves were collected for RAPD and ISSR analysis. For all experiments conducted, control explants were also cultured on MS media without any supplementation of plant growth regulator.

Statistical Analysis

All experiments had three replicates per treatment with each replicate consisting of a Petri plate with five explants, in a total of 15 explants per treatment. The experiments were repeated at least twice. The percentage data represented in the tables were arcsine transformed before being analysed for significance using analysis of variance (ANOVA; $P<0.05$). Further, the differences in means were contrasted using Duncan's new multiple range test following ANOVA. All statistical analysis was carried out using SPSS statistical software package version 16.0.

Assessment of the Genetic Fidelity

The genomic DNA from tender leaves (100 mg) of the eight randomly selected regenerated banana plantlets as well as from the mother plant maintained in the field was isolated by modified CTAB method [25]. The quantity of the extracted DNA was estimated using Biophotometer plus (Eppendorf, Germany) by taking their absorbance at 260 and 280 nm. RAPD-PCR amplifications were carried out with 40 RAPD primers (UBC set #5) performed in a Thermal Cycler (Bio-Rad, C1000™) with the following conditions: 3 min at 94 °C; 35 cycles of: 1 min at 94 °C, 1 min at 36 °C, 2 min at 72 °C and a final extension step 10 min at 72 °C. PCR reactions were carried out in a 20 μl reaction mixture volume containing 50 ng of template DNA, 100 μM dNTP mix (Himedia, India), 0.6 μM of random primers, 1× Taq DNA polymerase buffer containing 15 mM $MgCl_2$ (Sigma-Aldrich) and 1.0 U of Taq polymerase (Sigma-Aldrich Pvt. Ltd., Bangalore, India). The amplified products were resolved by electrophoresis on a 1.2 % (*w*/*v*) agarose gels run in 1× TBE buffer and detected by

ethidium bromide staining. The resulting fragments were scored under UV light using a gel documentation system (Bio-Rad, Australia) and analysed with Quantity One-1D software (Bio-Rad, Australia). A double-digested (EcoRI and HindIII) 1-Kb λ DNA ladder (Fermentas, USA) was used as molecular marker. All the PCR reactions were repeated at least twice to check the reproducibility. For the ISSR analysis, 30 primers (UBC set #9) were tested using similar amplification protocol with variable annealing temperatures and resolved on a 1.5 % (*w*/*v*) agarose gel. Only the distinct, reproducible and well-resolved fragments were scored as present (+) or absent (−).

Results and Discussion

In Vitro Regeneration

In the present study, the successful in vitro regeneration of *M. acuminata* (AAA) cv. Vaibalhla plantlets were achieved using immature male flower explants. Though the use of male flowers of banana is common for developing embryogenic suspension cultures or callus [26–28], limited reports have been found in the case of direct regeneration from immature male flowers [13, 14, 29].

The data on the effect of MS basal medium supplemented with different concentrations and combinations of PGRs viz. BAP, kinetin and NAA on immature male flower explants is given in Table 1. All explants showed varied morphogenetic response within 2 weeks of culture. The first change observed was the conversion of colour of the white immature male flowers to green, followed by bulging at the base. Bulging was not observed in explants cultured on MS medium supplemented only with 2,4-D and remained green (data not shown). Induction of WBLS was observed in the cultures by the third week with a maximum number (4.30) of WBLS per explant in MS media supplemented with a combination of BAP (2 mg L^{-1}) + NAA (0.5 mg L^{-1}) (Fig. 1e).

Table 1 Effect of plant growth regulators on immature male flower explants of *M. acuminata* (AAA) cv. Vaibalhla for WBLS formation

Plant growth regulators (mg L^{-1})			Time taken to form WBLS (weeks)	Percentage of explants forming WBLS (%) (mean ± SE)	Number of WBLS per explant (mean ± SE)
BAP	Kn	NAA			
2	0	0	10–12	33.33±2.35a	1.40±0.10a
3	0	0	7–10	8.88±2.15bk	1.16±0.16a
4	0	0	3–5	17.77±1.71 cl	2.05±0.24bj
5	0	0	3–5	31.10±1.39a	1.78±0.11aj
0	1	0	20–22	15.55±1.71dkl	2.16±0.16cj
0	2	0	7–9	24.44±1.50al	1.66±0.08aj
0	3	0	7–9	8.88±2.15ek	2.33±0.33dj
0	5	0	7–9	73.33±2.51fmn	2.06±0.16ej
2	0	0.5	5–7	71.10±1.39gmn	4.30±0.10f
4	0	0.5	5–7	35.55±2.71a	2.49±0.09gj
0	2	0.5	10–12	55.55±1.28ho	1.63±0.14aj
2	2	0	8–10	53.32±3.88io	2.12±0.01hj
3	3	0	5–7	62.22±1.32jno	2.10±0.11ij

Medium: MS + PGRs. Mean (±) followed by the same letter(s) in each column are not significantly different at $P<0.05$ using Duncan's new multiple range test

Table 2 Effect of plant growth regulators on shoot formation from buds derived from immature male flower explants of *M. acuminata* (AAA) cv. Vaibalhla after 16 weeks

Plant growth regulators (mg L^{-1})			WBLS forming shoots (%)	No. of shoots per explant (mean ± SE)	Length of shoots (cm) (mean ± SE)
BAP	Kn	NAA			
0	5	0	46.66±2.21a	1.69±0.10a	8.56±0.41a
2	0	0.5	48.88±1.27b	2.31±0.08b	14.44±0.15b
4	0	0.5	31.10±1.39c	1.21±0.01c	12.97±0.06c
0	2	0.5	77.77±1.51d	3.51±0.03d	10.50±0.06d

Medium: MS + PGRs. Mean (±) followed by the same letter(s) in each column are not significantly different at $P<0.05$ using Duncan's new multiple range test

Similar white buds were also observed from in vitro regenerated male flowers in different banana cultivars such as Sannachenkadali (AA), red banana (AAA) [13], dwarf cavendish [27], Virupakshi (AAB) and Sirumalai (AAB) [14]. The frequency and time taken for WBLS formation varied in MS media supplemented with different PGRs (Table 1). Buds primarily appeared within 2 months in earlier reports [14, 29] when cultured in high BAP concentrations (5–8 mg L^{-1}), while the present study shows the formation of WBLS only within 5–7 weeks in MS medium supplemented with a lower concentration of BAP (2 mg L^{-1}) + NAA (0.5 mg L^{-1}) in combination. Use of higher concentrations of BAP (4 and 5 mg L^{-1}) exhibited lesser time (3–5 weeks) to form WBLS. However, the total number of WBLS formation was much lesser and thus undesirable. Similar responses were observed in Berangan (AAA), Rastali (AAB), Nangka (AAB) and Abu (ABB) cultivars [29].

The induced buds were subsequently sub-cultured in MS media supplemented with combinations of different PGRs for shoot regeneration which responded with the formation of shoots within 2 weeks. The highest percentage (77.77) and number (3.51) of shoot formation was observed in MS media supplemented with a combination of kinetin (2 mg L^{-1}) and NAA (0.5 mg L^{-1}) (Table 2). Thus, BAP does not have a significant role in shoot formation, where MS media supplemented with BAP produced lesser number of shoots. These responses clearly indicate that though both BAP and kinetin are cytokinins (purine derivatives) causing similar effects in plants, their efforts on shoot proliferation vis-à-vis multiplication are different for cv. Vaibalhla (AAA). A similar kind of response was also observed in cv. Nanjanagudu Rasabale (AAB) [30].

The individual shoots were separated and sub-cultured in fresh media for elongation. MS medium supplemented with a combination of BAP (2 mg L^{-1}) and NAA (0.5 mg L^{-1}) showed the highest shoot length (14.44 cm). Appearance of leaves was observed from all the shoots

Table 3 Effect of plant growth regulators on root formation from shoots derived from immature male flower explants of *M. acuminata* (AAA) cv. Vaibalhla

Plant growth regulators (mg L^{-1})			Length of roots (cm) (mean ± SE)	No. of roots per plantlet (mean ± SE)
BAP	Kn	NAA		
0	0	0	20.23±0.04a	7.19±0.07a
0	5	0	17.85±0.28bf	5.28±0.12b
2	0	0.5	19.65±0.18c	4.64±0.08cf
4	0	0.5	17.30±0.06d	4.39±0.13df
0	2	0.5	18.18±0.13ef	4.33±0.07ef

Medium: MS + PGRs. Mean (±) followed by the same letter(s) in each column are not significantly different at $P<0.05$ using Duncan's new multiple range test

Table 4 Details of the RAPD profile obtained from genomic DNA isolated from in vitro regenerated plantlets of *M. acuminata* (AAA) cv. Vaibalhla

RAPD primer	Primer sequence (5′–3′)	Temperature (°C)	No. of monomorphic bands	No. of polymorphic bands	Total no. of bands amplified	Size range (bp)
UBC-418	GAGGAAGCTT	36	1	0	1	800–1,500
UBC-419	TACGTGCCCG	36	4	0	4	500–3,500
UBC-420	GCAGGGTTCG	36	2	0	2	800–3,000
UBC-421	ACGGCCCACC	36	2	0	2	800–3,000
UBC-433	TCACGTGCCT	36	3	0	3	100–3,500
UBC-438	AGACGGCCG	36	4	0	4	800–3,000
UBC-449	GAGGTTCAAC	36	1	0	1	100–800
UBC-450	CGGAGAGCCA	36	3	0	3	100–2,000
Total			20	0	20	

developed by the third week accompanied with root induction. For the evaluation of rooting efficiency in different media, the induced roots were cut off and the shoots were transferred to fresh media containing different PGRs. Within 1 week, fresh roots appeared in varied numbers and the maximum number (7.19) and length (20.23 cm) of roots was observed in the MS basal medium without any PGRs (Fig. 1g) followed by kinetin (5 mg L^{-1}) and the least with the combination of kinetin (2 mg L^{-1}) + NAA (0.5 mg L^{-1}) (Table 3). This shows that addition of auxin or cytokinins did not promote any significant increase in the rooting frequency in agreement with the earlier observations in AAB cvs. Virupakshi and Sirumalai [14].

The fully rooted plantlets were taken out from the culture bottles, washed thoroughly to remove any remains of medium, and planted in small plastic pots containing sterilised soil: sand (1:1). Plants were covered with transparent polyethylene bags to maintain adequate moisture for a week and transferred to the greenhouse (28 °C day, 20 °C night, 16 h day length and 70 % humidity). After a week, the plastic covering was removed and the plantlets were maintained in the greenhouse in plastic pots containing farm yard manure: sand

Table 5 Details of the ISSR profile obtained from genomic DNA isolated from in vitro regenerated plantlets of *M. acuminata* (AAA) cv. Vaibalhla

ISSR primer	Primer sequence (5′–3′)	Temperature (°C)	No. of monomorphic bands	No. of polymorphic bands	Total no. of bands amplified	Size range (bp)
UBC-827	$(AC)_8G$	47.5	4	0	4	800–3,500
UBC-829	$(TG)_8C$	47	4	0	4	800–2,000
UBC-830	$(TG)_8G$	47	4	0	4	500–2,000
UBC-834	$(AG)_8YT$	47	6	0	6	100–2,000
UBC-840	$(GA)_8YT$	47	5	0	5	100–1,500
UBC-843	$(CT)_8RA$	47	4	0	4	100–4,500
UBC-844	$(CT)_8RC$	47.5	5	0	5	800–3,000
UBC-845	$(CT)_8RG$	47	3	0	3	800–3,500
Total			35	0	35	

Where Y=C, T; R=A, G

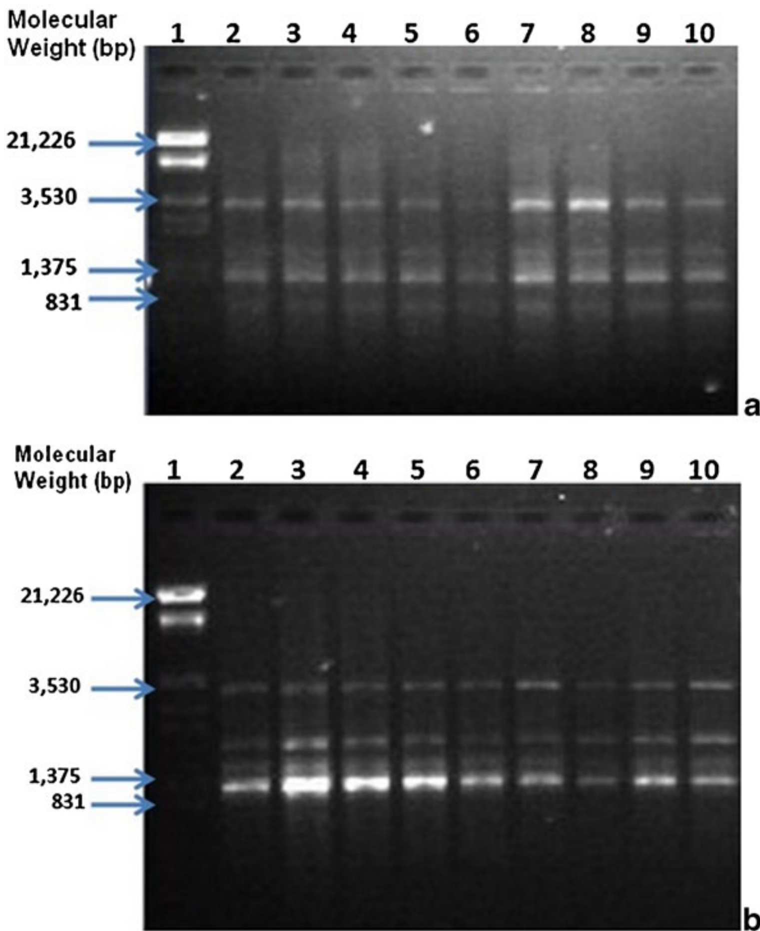

Fig. 2 RAPD and ISSR profiles of the in vitro regenerated plantlets derived from male floral explants of *M. acuminata* (AAA) cv. Vaibalhla. **a** Amplification with UBC RAPD Primer #419. **b** Amplification with UBC ISSR Primer #827. *Lane 1* lambda DNA HindIII marker, two donor plants, 3–10 regenerated plantlets

(1:1) (Fig. 1h) until they were transplanted to the nursery. About 96 % of the hardened plants survived in the nursery.

Analysis of the Genetic Fidelity of the In Vitro Raised Plantlets

True-to-true type clonal fidelity is one of the most important prerequisites in the micropropagation of any crop species. A major problem often encountered with the in vitro cultures is the presence of somaclonal variation amongst subclones of one parental line, arising as a direct consequence of in vitro culture of plant cells, tissues or organs. In banana micropropagation, though the protocol is known to yield clonal material, there are reports of somaclonal variation especially in cv. Williams [31]. A good analysis of genetic stability of plantlets can be made by using a combination of two types of markers that amplify different regions of the genome [11, 21, 32, 33]. Hence, in the present study, two PCR-based techniques, RAPD and ISSR, were adopted for evaluation of clonal fidelity in banana plantlets.

The RAPD and ISSR banding patterns of the eight randomly selected in vitro-derived plants compared with the donor mother plant confirm their genetic stability. Of the 40 RAPD primers tested, eight of them produced 20 distinct and reproducible amplicons in the size range of 100–3,500 bp, out of which all bands were present in mother plant as well as in all the sub-cloned plantlets in which none showed polymorphism (Table 4). The number of bands with each primer varied from 1 (UBC 418 and UBC 449) to 4 (UBC 419 and UBC 438) with an average of 2.5 bands per primer. On the other hand, after an initial screening of 30 ISSR primers, eight of them produced 35 clear and countable amplification products ranging between 100- and 4,500-bp lengths with no polymorphism. The optimum annealing temperature varied between 47.0 and 47.5 °C according to different ISSR primers (Table 5). Number of scoreable bands for each primer varied from 3 (UBC 845) to 6 (UBC 834) with an average of 4.37 bands per primer. The number of bands per primer was greater in ISSR (4.37) than RAPD (2.5). The representative profiles of the eight in vitro-derived plants and the control donor mother plant with RAPD primer (UBC 419) and ISSR primer (UBC 827) are shown in Fig. 2.

While genetic variations are exceptions, most of the organised cultures, especially the shoot tips maintain strict genotypic and phenotypic stability under tissue culture conditions [20]. This is also applicable to cv. Vaibalhla (AAA) since no variations were observed using the set of primers tested with the randomly selected regenerated plantlets. It has been found that high level of growth regulators caused variants like suppression of shoot length leading to morphological abnormalities and genetic changes in other genotypes of banana [10, 34]. Thus, in the present study, low levels of growth regulators were used to avoid generation of somaclonal variants.

The number of bands per primer was greater in ISSR (4.37) than RAPD (2.5). The reason for this could be because of high melting temperature for ISSR primers, which allows much more rigorous annealing conditions and consequently, more precise and repeatable amplification. Practical evidence is available showing that ISSR fingerprints detect more polymorphic loci when compared with RAPD fingerprinting [35, 36].

Conclusion

The present study describes an efficient method for in vitro plant regeneration of *M. acuminata* (AAA) cv. Vaibalhla, a very economically important banana variety of Mizoram for the first time. Clonally identical plantlets were regenerated from the immature male flowers obtained from the male bud. This method is cost-effective, where the male buds of banana were made useful in generating plantlets, thus meeting the demand for large number of elite suckers. The protocol will also be applicable for future conservation and genetic transformation studies of this commercially important banana.

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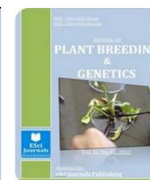

EVALUATION OF GENETIC DIVERSITY AMONG EDIBLE BANANA VARIETIES FOUND IN MIZORAM, INDIA USING RANDOMLY AMPLIFIED POLYMORPHIC DNA

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A B S T R A C T

Genetic variations in the edible non-seeded banana cultivars found in Mizoram, India was studied using RAPD markers. A total of seven edible cultivars belonging to different genome groups were analyzed using six standardized UBC-primers. Out of six primers used, two primers namely UBC 416 and 450 generates 100% polymorphism. The average polymorphism per primer was 92.7%. The polymorphism information content (PIC) of the primers in used ranged from 0.58 to 0.89. The resolving power of primer, UBC-418 was the highest (9.4) while that of primer UBC-419 was the lowest (3.7). The dendrogram analysis revealed the grouping of the cultivars into two major groups. The first group was constituted by all the six hybrid cultivars of A and B genomes while the second group was represented by the lone triploid AAA genome sample. The result of the principal co-ordinate analysis also supported the dendrogram grouping of the samples studied. Thus, the use of RAPD technique for the study of genetic variations in the banana cultivars was demonstrated. It was also shown that significant amount of genetic diversity existed in the samples studied, thereby providing clear genetic information for future breeding and conservation strategies.

Keywords: Edible banana, Mizoram, RAPD, genetic variation.

INTRODUCTION

Banana is regarded as one of the favorite fruit crops of the world and is widely distributed throughout the tropics in more than 120 countries with a total production of approximately 106 million tonnes per year (Molina and Kudagamage, 2002). It is the fourth most important crop after rice, wheat, and maize (Uma and Sathiamoorthy, 2002). Banana originated from southeast Asia where they occur from India to Polynesia (Simmonds, 1962; Sathiamoorthy *et al*., 2001; Daniells et al., 2001). Nearly half of the world banana production is cultivated in Asia, while almost 75% of the world's plantains are cultivated in Africa. Edible banana along with ornamental ones and plantains belong to the family of *Musaceae*, which consists of two genera: *Musa* and *Ensete*. The taxonomy of the approximately 50 species within the genus Musa remains poorly resolved, not least because of the widespread vegetative reproduction and natural occurrence of many hybrids (Heslop and Schwarzacher, 2007). Most frequently, the genus is divided into four (sometimes five) sections, Eumusa and Rhodochlamys with a basic chromosome number of x = 11, Australimusa (x = 10), and Callimusa (x = 10 or x = 9) (after Cheesman, 1947; Simmonds and Weatherup, 1990; Dolezel and Bartos, 2005). Various minor and major regroupings have been suggested (Wong et al., 2002). At the species level, the number of species and the status of subspecies have been debated (Taxonomic Advisory Group for Musa, 2007). Eumusa constitutes the sources of edible banana chiefly belonging to *M. acuminata* Colla and *M. balbisiana* Colla originating from the inter- and intra-specific hybridization (Ashalatha *et al*., 2005). Depending on the contribution of *M. acuminata* (AA) and *M. balbisiana* (BB), the cultivars have been classified into genomic groups (AA, AAA, AAB, ABB, BB, AB, BBB, AAAA and ABBB). The cultivated edible bananas and plantains differ from their wild relatives by being seedless and parthenocarpic. The genetic basis of the mutation (or mutations) in the A genome that gives rise to parthenocarpy has not been characterized, and no parthenocarpy has been identified

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in B genome diploids, although hybrids of A and B show the character (Heslop and Schwarzacher, 2007). Most of

the cultivars are wild collections made by farmers of spontaneously occurring mutants with parthenocarpic fruit production, which were brought into cultivation and then multiplied and distributed by vegetative propagation. India is the leading country in global banana production with an annual production of 13.5 mt from an area of 4.0 lakh ha and is further focusing to achieve 25 mt by the end of 2020 in production level (Sathiamoorthy *et al*., 2001). India has a rich genetic diversity of banana with more than 90 distinct clones. The northeastern region of India have been considered as the richest sources of natural banana diversity where *M. balbisiana* from Indian subcontinent meet *M. acuminata* from South East Asia (Molina and Kudagamage, 2002). However, the vast genetic resource of wild and cultivated banana in the region remains unexplored and untapped for scientific studies. Mizoram is located in the northeastern corner of India, enjoying the tropical, sub-tropical and temperate region in the state. Wild and edible banana varieties are abundantly distributed throughout Mizoram. Therefore, the understanding of the level of genetic diversity of edible banana in the state will provide useful information for the status and breeding purposes.

Morphological characters have been used for classifying genome constitution and ploidy levels in banana (Pollefeys et al., 2004). However, phenotyping is difficult because of the size of the plants, and long life cycle and thus molecular marker methods are widely used for germplasm characterization. RAPD, AFLP and microsatellites (Onguso *et al*., 2004; Wong *et al*., 2002) have been used for the characterization of banana. Uma *et al*., (2005) have also reported the evidence of genetic variation successfully in Indian wild *Musa balbisiana* (BB) population by using RAPD markers. The use of RAPDs in molecular biology has the advantage that the material is processed by an efficient and inexpensive technique without requiring prior knowledge of the genome (Bhat *et al*., 1995). RAPD assay has the advantage of being easy to use, requiring very small amount of genomic DNA without the need for blotting and radioactive detection and are moderately reproducible (Cipriani *et al*., 1996). Thus, the present study was undertaken to analyze the genetic variation of edible cultivated banana varieties maintained in the field gene bank of the Department of Biotechnology, Mizoram University using RAPD markers.

MATERIALS AND METHODS

Plant Material: For the present study, seven cultivated non-seeded edible banana cultivars maintained in the field gene bank of the Department of Biotechnology, Mizoram University, Aizawl were selected (Table 1).

Table 1 List of edible non-seeded banana cultivars of Mizoram chosen for the study.

Accession (Local name)	Code	Species	Genome group
Vaibalhla	VB	*Musa acuminata*	AAA
Banria	BR	*Musa sp.*	ABB
Lawngbalhla	LB	*Musa sp.*	AAB
Kawlbalhla	KB	*Musa sp.*	ABBB
Banthur	BT	*Musa sp.*	AAB
Banpawl	BP	*Musa sp.*	ABB
Balhlasen	BS	*Musa sp.*	AAB

DNA extraction and PCR amplification: The young cigar leaves were collected from the banana plants and used for DNA extraction. Leaves weighing approximately about 200 mg were used in the DNA extraction using the modified CTAB method protocols of Thangjam *et al.* (2003).

The quantity of the extracted DNA was estimated using Biophotometer plus (Eppendorf, Germany) by taking their absorbance at 260 nm and 280 nm. For each sample, a 100 ng/µl stock was prepared from the original mother stocks for further analytical procedures. The PCR reaction mixture was assembled in a 25 µl volume containing 50 ng of template DNA, 1X Taq buffer, 0.5 µM dNTPs, 2 µM $MgCl_2$, 1U Taq polymerase (Bangalore Genei Pvt. Ltd., India) and 50 pmol UBC RAPD primer set # 5 (University of British Columbia, Canada). The thermocycler (Applied Biosystems, USA) was programmed at 1 cycle of 94 ^{0}C for 1 min and then 35 cycles of 94 ^{0}C for 1min 36 ^{0}C for 50 sec and 72 ^{0}C for 2 min. The last extension cycle was programmed at 72 ^{0}C for 4 min. The amplified products were electrophoresed on a 1.8% ethidium bromide stained agarose gel in TBE buffer and then photographed using a gel documentation system (UVP Ltd., UK).

Analysis of the RAPD profiles: The amplified DNA fragment generated was treated as a separate character and scored as a discrete variable, using 1 to indicate presence, and 0 for absence of DNA fragments. Accordingly rectangular binary data matrix was generated from the RAPD profiles.

Primer banding characteristics such as number of scored bands (NSB), number of polymorphic bands (NPB), and percentage of polymorphic bands (PPB) were obtained. Resolving power and marker index for the primers were also calculated. Polymorphism information content (PIC_i) of a band was calculated according to Anderson *et al.* (1993) as follows:

$$PIC_i = 1 - \sum_j f_{ij}^2$$

Where f_{ij} is the frequency of the jth pattern of the ith band (note that dominant markers have two patterns for a band as being present and absent). Then, the PIC of each primer was calculated as:

$$PIC = 1/n - \sum_{i=1}^{n} PIC_i$$

where n is the NPB for that primer. Informativeness of a band (BI_i) was calculated as:

$BI_i = 1 - (2 \times | 0.5 - p |)$

where p is the proportion of the seven accessions containing the band. Then, the resolving power (RP) of each primer was calculated as:

$$RP = \sum_{i=1}^{n} IB_i$$

where n is the NPB for that primer (Prevost and Wilkinson, 1999). Further we calculate mean resolving power for each primer as:

MRP = 1/n RP

Following Milbourne et al. (1997), marker index (MI) was calculated as product of PIC and effective multiplex ratio (EMR), which is defined as the product of the fraction of polymorphic loci and the number of polymorphic loci (Seyit *et al.,* 2010).

Statistical analysis: Rectangular binary data matrix of RAPD was used for statistical analyses. Pairwise dissimilarity matrix was generated using Nei's coefficient (Nei, 1978) and principal coordinate analysis (PCoA) of the doubled centre distance matrix was performed. A dendrogram using neighbor joining method (unweighted) was constructed on the basis of the dissimilarity matrix data. All the analysis was conducted using the software NTSYS-pc version 2.20a (Numerical Taxonomy and Multivariate Analysis for Personal Computer, Rohlf, 2000).

RESULTS AND DISCUSSION

The present study was carried out to evaluate the level of genetic variations in the cultivated non-seeded banana cultivars of Mizoram using RAPD markers. The details of the RAPD profile generated in the banana samples studied using 6 RAPD primers were given in Table 2. Only the distinct and reproducible bands were scored for the analysis. A total of 94 fragments were scored out of which 84 were polymorphic (92.5%). This amplification result indicates that the randomly arranged nucleotides sequences in UBC- primers have their complementary nucleotides with the isolated DNA from the samples. The patterns of their dissimilarity in amplifications signify that they are genetically asymmetric. Selective amplification products in PCR condition by using random primers established that the tested DNA samples were diversified from one another. Different samples produce different numbers of bands with a particular primer. Two primers UBC-416 and -450 generated 100% polymorphism. RAPD profile generated by the UBC-416 was represented in Fig. 1.

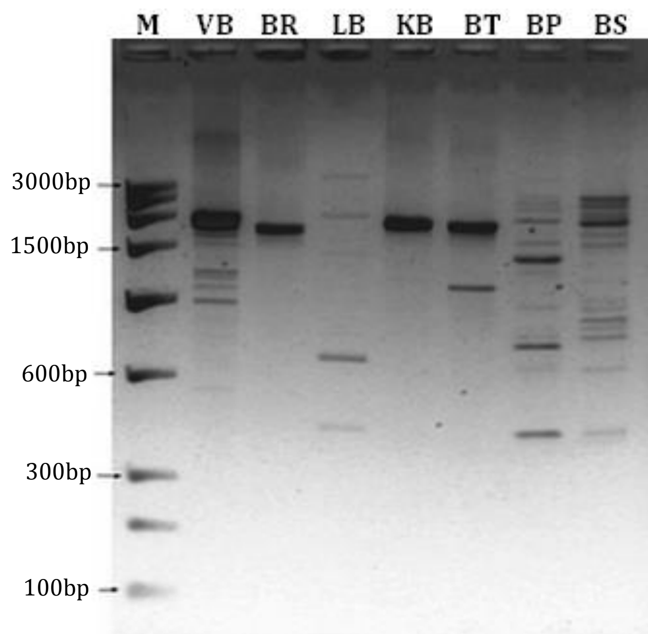

Fig. 1 RAPD profile generated by the primer UBC-416 (5´-GTGTTTCCGC-3´) in the banana samples studied. M- Low range DNA Ladder; VB- Vaibalha; BR- Banria; LB- Lawngbalha; KB- Kawlbalha; BT- Banthur; BP- Banpawl; BS- Balhlasen.

The average polymorphism was found to be 92.7%. The polymorphism information content (PIC) for the RAPD primer, UBC-416 was the highest (0.894) while that of primer UBC-421 was the lowest (0.582). The mean resolving power (MRP) of the primers ranges from 0.46 (UBC-419) to 0.63 (UBC-418). Resolving Power (Rp) provided a modest indication of the ability of these primers to distinguish between cultivars. Marker index (MI) which is the discriminating power for a marker was also calculated for each primer and found to be highest in the primer UBC-416 (16.1). Rp of a primer has been found to correlate strongly with its ability to distinguish between cultivars. The function is well suited for comparing primers, primer-enzyme combinations or probe-enzyme combinations generated by RAPD, ISSR-PCR, AFLP or RFLP analyses. Crucially, Rp provides quantitative data allowing comparisons between primers (or probe-enzymes etc.), including those that are able to distinguish all genotypes examined in a study. It can also be used to predict the performance of groups of primers.

Table 2 Details of amplification bands and their associated polymorphic fragments obtained with different UBC decamer oligonucleotides utilized as random primers in the banana samples. NSB- Number of scored band; NPB-Number of polymorphic band; PPB-Percentage of polymorphic band; PIC- Polymorphism information contents; RP-Resolving power; MRP- Mean resolving power; MI- Marker index.

UBC Primer (set # 5)	Sequence (5´ - 3´)	Fragment size range (bp)	NSB	NPB	PPB	PIC	RP	MRP	MI
UBC-416	TGTTTCCGC	2000-350	18	18	100	0.894	8.5	0.47	16.1
UBC-418	AGGAAGCTA	2000-300	16	15	93.7	0.758	9.4	0.63	10.6
UBC-419	ACGTGCCCG	2700-440	9	8	88.9	0.775	3.7	0.46	5.51
UBC-420	CAGGGTTCT	2400-270	15	14	93.4	0.821	8.3	0.59	10.7
UBC-421	CGGCCCACC	3000-400	20	16	80	0.582	8.8	0.55	7.45
UBC-450	GGAGAGCCA	2000-450	16	16	100	0.665	8.0	0.50	10.6

The Nei's (1978) dissimilarity matrix between the samples was given in Table 3. The highest similarity was obtained between the two cultivars (BR and BP) belonging to the ABB genome with a dissimilarity index of 0.156 and the lowest similarity was found between the AAA cultivar (VB) and ABB cultivar (BP) with a score of 0.754.

Table 3 Dissimilarity matrix based on Nei's coefficient (1978) obtained from RAPD profile in the banana samples studied. VB- Vaibalha; BR- Banria; LB- Lawngbalha; KB- Kawlbalha; BT- Banthur; BP- Banpawl; BS- Balhlasen.

Sample	VB	BR	LB	KB	BT	BP	BS
VB	0.00						
BR	0.739	0.000					
LB	0.724	0.455	0.000				
KB	0.647	0.468	0.693	0.000			
BT	0.668	0.346	0.559	0.531	0.000		
BP	0.754	0.156	0.449	0.618	0.324	0.000	
BS	0.611	0.467	0.734	0.583	0.474	0.376	0.000

The scores obtained from the RAPD profiles were used to construct a dendrogram using unweighted method of the Neighbor Joining cluster analysis. The resulted dendrogram revealed the clustering of the seven samples into two distinct groups (Fig. 2). The first group was sub-divided into 2 clusters, with 3 cultivars namely – KB (ABBB genome), BS (AAB genome) and BT (AAB genome) in the first cluster and another 3 cultivars namely – LB (AAB genome), BP (ABB genome) and BR (ABB genome) in the second cluster. The lone AAA genome sample (VB) was place in the second group.

The analysis of the principal co-ordinate analysis was represented by the 3-D projection diagram (Fig. 3). The extracted co-ordinates was also in accordance with the dendrogram, projecting VB alone in one different dimension while BR, BP and LB were clustered together and likewise BT, BS and KB also form one cluster.

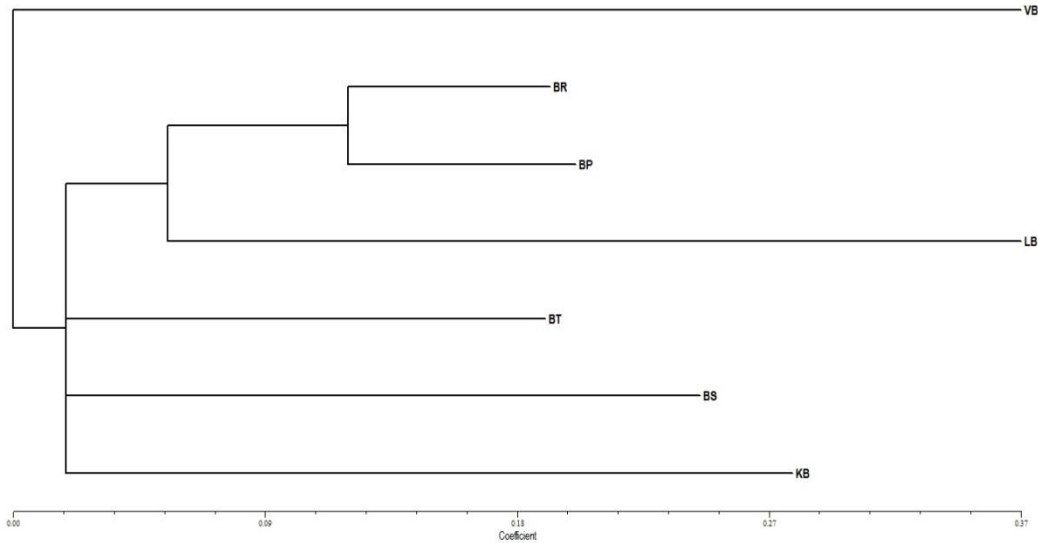

Fig. 2 Dendrogram resulted from the analysis of RAPD data. VB- Vaibalha; BR- Banria; LB- Lawngbalha; KB- Kawlbalha; BT- Banthur; BP- Banpawl; BS- Balhlasen.

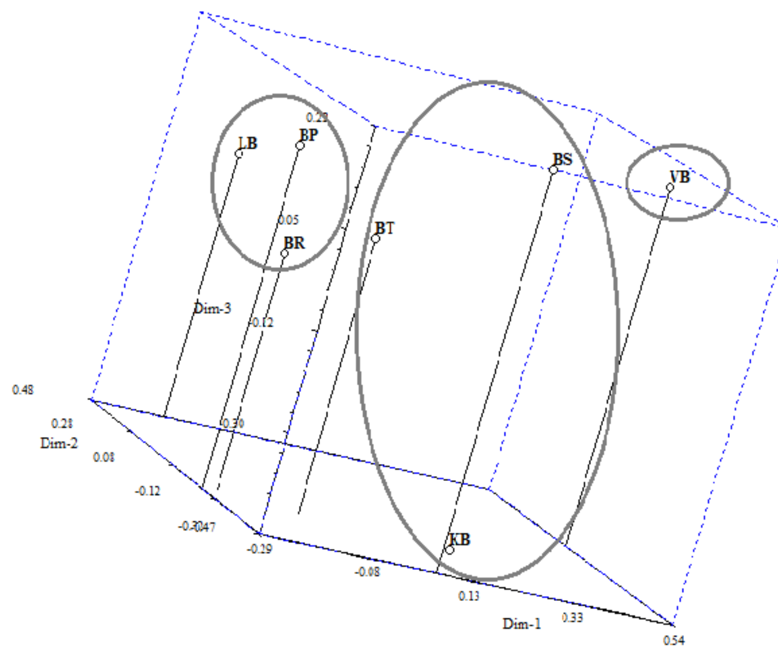

Fig.3 Three-dimensional projection of the RAPD profile generated by the banana samples studied. VB- Vaibalha; BR- Banria; LB- Lawngbalha; KB- Kawlbalha; BT- Banthur; BP- Banpawl; BS- Balhlasen.

The present study was primarily focus on the study of genetic variation among the edible non-seeded banana varieties from locally available banana in Mizoram. Using RAPD technique it was shown that significant amount of genetic diversity existed in the samples studied. Therefore, understanding genetic variation between and within the cultivars is very important for the evaluation of plants with superior traits. The finding derived out of this study will provide useful information for future breeding and conservation strategies of banana genetic resources in the northeast region of India, which is considered as a storehouse of diverse gene-pool.

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DNA barcoding as a tool for rapid identification of plants: a case study in banana family (Musaceae)

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ABSTRACT

DNA barcodes are unique sequence patterns of small DNA fragments that are used as specific reference collections to identify specimens and to discover overlooked species. DNA barcoding provides rapid, accurate and automatable species identification using standardized DNA sequences. The most important characteristic features of a DNA barcode are its universality, specificity on variation and easiness on employment. Thus the present investigation was conducted to evaluate the ITS2 sequences of the nuclear ribosomal DNA and the chloroplast rbcL gene sequences for possible DNA barcoding of the members of the Musaceae family. Successful PCR amplification of the ITS2 and rbcL regions in the 6 banana samples studied was achieved using the universal primers. The genetic sequences derived from the PCR products revealed the variable length and topology. Homology search using Blast resulted in the high similarity values of 97-99% in ITS2 and 95-99% in rbcL between the sequences generated and the Genbank databases. Using 2 reference sequences of the genus Ensete, the sequences obtained from the banana samples were analysed. The overall analysis of the sequences revealed the phylogenetic relationship based on the sections and the classification of Musaceae. The ITS2 sequences revealed the superiority in in terms of detecting the inter- and intra-species variations over the rbcL sequences. However, the DNA barcoding of the members of the Musaceae family could not be achieved and further works on the use of additional markers and members of the family are required. The finding therefore provides glimpses of the possibility of ITS2 as a possible DNA barcode at the interspecies level.

Key words: DNA barcoding, Phylogeny, ITS2, rbcL, Musaceae.

INTRODUCTION

DNA barcoding is a relatively new concept that has been developed for providing a rapid, accurate and automatable species identification using standardized DNA sequences as tags. In fact, it started with the seminal work of Hebert *et al.,*(2003). DNA Barcoding is the process of identification of species based on nucleotide diversity of short DNA segments. However, in plants, due to the difficulty in finding a universally acceptable barcode.it is yet to be well established. Based on the relative efficacy testing, the Consortium for the Barcode of Life-Plant Working Group has recently identified a few loci as potential barcode candidates and from them a two-locus standard barcode (*rbcl+matK*) has been recommended for initiating the barcoding

process of plant species. In DNA barcoding, the unique nucleotide sequence patterns of small DNA fragments (400-800 bp) are used as specific reference collections to identify specimens and to discover overlooked species. Thus, the initial goal of the DNA Barcoding process is to construct on-line libraries of barcode sequences for all known species that can serve as a standard to which DNA barcodes of any identified or unidentified specimens can be matched. This can alleviate several inherent problems associated with traditional taxonomic identification, based on morphological characters, such as wrong identification of species due to phenotypic plasticity and genotypic variability of the characters, overlooking cryptic taxa, difficulty in finding reliable characters due to long maturity periods. DNA barcoding thus provide the taxonomists, conservationists and others who need the identification of species, a cost-effective tool, much as a barcode that identifies supermarket products. It is especially of much use in areas where species identification with morphological characters is not practicable due to extensive damage or delayed expression. With the support of CBOL, the effort of DNA barcoding has been slowly progressing with controversies and intense debates.

Evaluation of Nuclear and Chloroplast gene sequences for feasibility of DNA Barcoding of Musaceae family.

Bananas and plantains belongs to the family Musaceae Jussieu (1789), distributed highly in tropical and sub-tropical regions in the world found growing in more than 120 countries. *Musa* L. (Linnaeus 1753) is one of the three recognized genera of the family Musaceae along with *Ensete* Horaninow (1862) and *Musella* (Fr.) . The genus *Musa* originated from Southeast Asia and consists of around 65 species while *Ensete* is a small genus of 8-9 species distributed in Asia, Sub-Saharan Africa and Madagascar and *Musella* is a monotypic genus native to Southwest China (Cheesman1947; Baker and Simmonds 1953). According to Cheesman (1947), *Musa* is divided into four sections based on the phenotypic traits and basic chromosome numbers, such as Eumusa (x = 11), Rhodochlamys (x = 11), Australimusa (x = 10) and Callimusa (x = 10). Recently Wong *et al*., (2002) divided *Musa* into five sections with the addition of Ingentimusa (x = 7). Among the five *Musa* sections, Eumusa contributed the largest number, most diversified and ancient including the two wild diploid progenitor species of *M.acuminata* Colla (A genome) and *M. balbisiana* Colla (B genome) (Bekele and Shigeta 2011). According to Simmonds and Shepherd (1955) the inter and intra specific crosses between these two species leads to the existence of different varieties with various genome composition resulting several ploidy level ranging from diploids to tetraploids (AA, AB, AAB, ABB, ABBB, AABB etc.) and posing a huge challenge towards its proper identification.

According to Li *et al*. (2010) Musaceae contains only two genera *Musa* L. (Linnaeus, 1753) and *Ensete* Horan (Horaninow, 1862). The genus *Ensete* differs from *Musa* by being monocarpic, non-suckering (except *E. ventricosum*) with a distinctively swollen base and having large-sized seeds while *Musa* produces suckers and has small seeds (Samson, 1992). However, the clarity on the taxonomy of *Ensete*remains a subject of debate and so far 6 species have been identified (Bekele and Shigeta, 2011). They are distributed in Asia, sub-Saharan Africa and Madagascar (Cheesman1947; Baker and Simmonds, 1953). A few species are also reported from northeast India to the Philippines and New Guinea (Purseglove, 1972). The genus *Musa* contains 30-40 species and the taxonomy is based on plant phenotype, flower orientation and arrangement and also the basic chromosome number (Ude et al., 2002).

Origin and distribution of banana

The *Musa* domestication process started some 7,000 years ago in Southeast Asia (D’ Hont *et al*., 2012). It involves the hybridization between diverse species and subspecies, cultivated by human migrations (Perrier *et al*., 2011) and se-

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lection of diploid and triploid seedless, parthenocarpic hybrids thereafter widely dispersed by vegetative propagation. Simmonds (1962) described Southeast Asia as the centre of origin of banana and plantains and the majority of cultivated bananas arose from the *Eumusa* section. This section is the largest, most geographically diversified with ~21 species and mainly distributed in the Indo-Malaysian and Southeast Asian countries to North Australia (Li *et al*., 2010). It also consists of the two wild diploid progenitor species namely - *M. acuminate* Colla(A genome) and *M. balbisiana* Colla(B genome) which leads to the origin of different genome combinations (AB, AAB, ABB and ABBB etc.) through intra and inter-specific crosses (Uma *et al*., 2006). Azhar (2006) stated that either Malaysia (Simmonds, 1962) or Indonesia (Horry *et al*., 1997) is the centre of diversity of *M. acuminata* whereas *M. balbisiana* is originated from India, Myanmar, Thailand and Philippines. Few of the edible hybrids originated from crosses between *M. acuminata* and *M. schizocarpa* (S genome) as well as from *M.acuminata* and *M. textilis* (T genome) have also been reported (Hřibová *et al*., 2011). *M.acuminata* is the most widespread of the *Eumusa* and the occurrence of spontaneous mutation, or recombination events have resulted the development of natural reproductive barriers within the species, causing subspecies divergence and genetic diversity in the species as a whole. In addition, edibility of mature fruits of diploid *M. acuminata* (AA) resulted due to female sterility and parthenocarpy (Daniells *et al*., 2001). Initially these spontaneously occurring mutant edible cultivars with parthenocarpic fruits were domesticated by farmers and brought into cultivation. From the domesticated stocks of the cultivated bananas further distribution and propagation took place by vegetative means through suckers (Heslop-Harrison and Schwarzacher, 2007). However, the genetic basis of the mutation (or mutations) in the 'A genome' that gives rise to parthenocarpy has not been characterized so far and no parthenocarpy has been identified in 'B genome' diploids, although hybrids of A and B show the character.

The cultivated bananas and plantains differ from their wild relatives by being seedless. Fruits of these cultivated species are typically sterile or have extremely low fertility. Majority of the cultivated bananas are triploid ($2n = 3x = 33$) with a minor contribution of diploid and tetraploid ($2n = 4x = 44$) (Heslop-Harrison and Schwarzacher, 2007). The hybridization events and mutations giving rise to the seedless and parthenocarpic characters have been occurring many hundreds of times in the wild and wherever the fertile plants occur together, hybridization continues to produce new recombinations and parental lines (Pollefeys *et al*., 2004). The hybridization of *M. acuminata* from southeast Asia with *M. balbisiana* from Indian subcontinent occcured mainly due to human intervention leading to the evolution of AB, AAB and ABB cultivars which are greatly diversified in the region (Daniells *et al*., 2001). The early dispersal of such banana cultivars resulted in the development of distinct subgroups of varieties in different geographic locations. Secondary diversification within the major sub-groups of cultivated bananas is thought to have been the result of somatic mutations rather than sexual reproduction. Mutations affecting traits of economic or horticultural interest have been selected by farmers over the years and multiplied by vegetative propagation to produce morphotypes.

Status of research on banana

Banana and plantains (*Musa* spp.) are one of the top world trade commodities and as food security for millions of people in the tropical and sub-tropical regions. It is the fourth most important food crops (after rice, wheat, and maize), highly distributed in more than 120 countries with an annual production of 106 million tonnes (FAO, 2005). It originated from Southeast Asia and then diversified widely across the world (Simmonds and Shepherd, 1955). India is the largest producer of banana in the world with annual production of 13.5 mt from an area of 4.0

lakh (Daniells *et al*., 2001). Besides the cultivated species and their cultivars, majority of the species of *Musa* were found in wild condition and they are widely distributed in the northeast states. The northeastern region of India have been considered as the richest sources of natural banana diversity where *M. balbisiana* from Indian subcontinent meet *M. acuminata* from South East Asia (Molina and Kudagamage, 2002). Uma *et al*. (2001) had made the survey and recorded 13 different bananas and plantains of Mizoram, Northeast India.

For the improvement of banana production and the planting materials at the global level, International Network for the Improvement of Banana and Plantain (INIBAP) and other international research centers were established to emphasize on banana research with the main objective of sustainable production of banana and plantain for domestic consumption and for local and export markets. Sathiamoorthy *et al*. (2001) reported that the first improvement programmed on banana in India was started in 1949 at Central Banana Research Station in Aduthurai in Tamil Nadu. Its main objectives were collection, conservation and evaluation of *Musa* germplasm and identifying accessions resistant/tolerant to stress by screening their reaction to pests and diseases for sustainable production. Of the 8 banana genebanks in India, National Research Centre on Banana (NRCB), Trichy, Tamil Nadu which was established by the Indian Council of Agricultural Research (ICAR) in 1993 has now a collections of more than 970 indigenous banana from all banana growing areas including southern state, Gujarat, Meghalaya, West Bengal, Bihar. Among these banana 109 were collected from Northeast states (Assam, Meghalaya, Arunachal Pradesh, Mizoram, Tripura and Sikkim). Assam Agricultural University (AAU) in Kahikuchi and Jorhat are the two banana gene banks available in Northeast India where there are 87 and 96 numbers of accessions respectively. Independent collections are also being maintained in the institutes of northeast region such as Mizoram, Arunachal Pradesh, Manipur, Assam, etc.

Molecular markers:

Details of the used molecular markers for the evaluation of nuclear and chloroplast gene sequences for feasibility of DNA barcoding of banana are explained as follows:

Internal Transcribed Spacers regions of nuclear ribosomal cistron: The rDNA cistron is a multigene family encoding the nucleic acid core of the ribosome.Within the cell, the rDNA is arranged as tandemly repeated units containing *18S,* 5.8*S*, 26*S* coding regions and two internal transcribed spacers (ITS1 & ITS2) present on either side of 5.8*S* region. Generally, the rDNA units are reiterated thousands of times and are organized into large blocks in the chromosome called the nuclear organizer regions. One of the most remarkable features of the rDNA is that the individual unit of this multiple gene family does not evolve independently, instead all the units evolve in a concerted manner such that higher level of overall sequence homogeneity exists among copies of the rDNA within a species, but differs among different species. This high sequence homogeneity is achieved through a process initially termed as horizontal evolution or coincidental evolution, but later renamed as concerted evolution which involves unequal crossing over and gene conversions. Currently, *nr*ITS is considered as one of the most useful phylogenetic markers for both plants and animals, because of its ubiquitous nature, biparental inheritance, and comparatively higher evolutionary changes due to less functional constrains. Likewise species-level discrimination and technical ease have also contributed to its wider acceptability as a powerful phylogenetic marker. Another advantage is that the ITS1 and ITS2 regions can be PCR-amplified separately by anchoring primers in the conserved coding regions. This facilitates easy amplification of ITS even from poor quality or degraded DNA. Universal primers are also available for amplification of ITS1 and ITS2 regions.

rbcL gene sequence: Among the plastid genes, rbcL is the best characterized gene se-

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quence. Therfore, most of the investigating groups tested its suitability in barcoding. It encodes the large subunit of rubilose-1.5-biphosphate carboxylase/oxygenase (RUBISCO) . As RUBISCO is a critical photosynthetic enzyme, rbcL was the first gene that was sequences from the pants. rbcL has been used so extensively in plant phylogenetic studies that more than 10,000 rbcL sequences are already available in GeneBank. Because of this wide utility, various aspects of the molecular evolution of rbcL have also been studied in detail. Most of the phylogenetic studies suggest that rbcL is best suited to reconstruct the relationships down to the generic levels, but is not useful for species level. Furthermore, inorder to obtain enough species discrimination,, the entire ~1430bp needs to be sequenced, which acts as a limiting factor for its use as a barcoding sequence because an ideal DNA barcoding region should be sort enough to amplify from degraded DNA and analysed via single-pass sequencing. Primers for PCR amplification and sequencing for such short sequence within the rbcL gene have been developed accordingly for most of the taxa. Owing to the ease in PCR amplification across a wide range of plant groups and the availability of sequence information in many plant groups, the CBOL-Plant Working Group has recently recognized rbcL as one of the most potential gene sequences for DNA barcoding in plants. However, because of the low species discrimination. Most of the investigating groups are of the opinion that rbcL should be used in conjunction with other markers. Therfore, CBOL-Plant Working Group recommended a combination of genes appears to be pragmatic solution to a complex trade-off among universality, sequence quality discrimination and cost. Thus the present investigation was carried out to test the universality of the nuclear and chloroplast gene sequence loci in Musaceae family and evaluate the feasibility of DNA barcoding.

MATERIALS AND METHODS

Plant materials: For the present study six (6) different banana accessions maintained in the field gene bank of Biotechnology Department, Mizoram University were selected. The details of the samples are given in Table 1.

DNA extraction:

Fresh young cigar leaves were collected from each plant maintained in the field gene-bank of the Department of Biotechnology, Mizoram University, Aizawl which represent the respective voucher number. The plant materials were subsequently used for the DNA extraction in the present study. Extraction of genomic DNA was carried out with the modified protocols of Thangjam *et al*. (2003) with minor modifications.

PCR-amplification of the ITS2 region

For the PCR-amplification of the ITS2 region, genomic DNA of each of the 6 banana cultivars were amplified with ITS 2 primer (Chen *et al.,2010*) having the forward sequence of 5´-ATGTCACCACAAACAGAAAC-3´ and having the reverse sequence of 3´-GACGCTTCTCCAGACTACAAT-5´ purchased from Eurofins Genomics Pvt. Ltd., Bangalore, India. The PCR amplification was carried out on a Gene AMPPCR System 9700 (Applied Biosys-

Table 1: List of banana plants used for the study

Sl. No.	Voucher no.	Name of the species	Section
1.	MZUTRS-13	*Musa* ABB group cv. Banpawl	Eumusa
2.	MZUTRS-14	*Musa* AAB group cv. Balhlasen	Eumusa
3.	MZUTRS-01	*Musa balbisiana* BB group cv. Changthir	Eumusa
4.	MZUTRS-08	*Musa ornata* cv. Changvandawt	Rhodoclamys
5.	MUTRS-28	*Musa laterita* cv. Red	Rhodoclamys
6.	MUTRS-29	*Musa laterita* cv. Green	Rhodoclamys

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tems, USA) with 25 µl reaction mixture containing 1× PCR buffer, 100 ng of genomic DNA as template, 0.5 µM each of the primers, 1.5 mM of $MgCl_2$, 0.2 mM of dNTP, 1 U of Taq DNA polymerase(Bangalore Genei, India). The PCR reaction was set up with the condition of 94°C for 4 min, 35 cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 1 min followed by a final extension at 72°C for 7 min. The amplified fragments were resolved on a 1.5% agarose gel and the gels were stained with ethidium bromide and visualized under UV light with a 100 bp DNA ladder (Bangalore Genei, India) as a marker and then photograph using a gel documentation system.

PCR-amplification of the rbcL region

For the PCR-amplification of the rbcL region, genomic DNA of each of the 6 banana cultivars were amplified with rbcL primer (Chen *et al.,2010*) having the forward sequence of 5´-ATGTCACCACAAACAGAGACTAAAGC-3´ and having the reverse sequence of 3´-CTTCTGCTACAAATAAGAATCGATCTC-5´.The PCR amplification was carried out on a GeneAMPPCR System 9700 (Applied Biosystems, USA) with 25 µl reaction mixture containing 1× PCR buffer, 100 ng of genomic DNA as template, 0.5 µM each of the primers, 1.5 mM of $MgCl_2$, 0.2 mM of dNTP, 1 U of Taq DNA polymerase(Bangalore Genei, India). The PCR reaction was set up with the condition of 95°C for 4 min, 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min followed by a final extension at 72°C for 7 min. The amplified fragments were resolved on a 1.5% agarose gel and the gels were stained with ethidium bromide and visualized under UV light with a 100 bp DNA ladder (Bangalore Genei, India) as a marker and then photograph using a gel documentation system.

Sequencing of the PCR fragments

The desired ITS2 and rbcL PCR fragments of 560 bp and 680 bp respectively generated by the six samples were sent directly for sequencing to GCC Biotech Pvt. Ltd. Kolkata, India.

Sequence analysis

The raw sequences obtained were first checked for its accuracy and edited by comparing with the associated chromatogram using Bioedit software ver. 7.5.1.0 (Hall, 1999). Homology search of the sequences was performed using the Blastn (Altschul et al., 1990) against the nucleotide GenBank database (http://www.ncbi.nlm.nih.gov) using the megablast program to search for the homologous sequences. The identified and annotated sequences were aligned using the multiple sequence alignment tool of the DNAMAN software package ver. 6.0.3 (Lynnon, Quebec, Canada). Analysis of nucleotide composition, Tajima's neutrality test (Tajima, 1989), pairwise distance (Tamura and Nei, 1993) and phylogenetic analysis using maximum likelihood method using MEGA software ver 5.0.(Tamura et al., 2011). Evaluation of the ability of the ITS2 and the rbcL gene sequences to analyse the inter-species divergence were performed using Wilcoxon signed-rank test tool of the SPSS software ver. 16.0. For all these analyses 2 species of the genus *Ensete* viz., *E. glaucum* and *E. ventricosum* were taken as an outgroup with their sequences (both ITS2 and rbcL) retrieved from the genbank database (www.ncbi.nlm.nih.gov/genbank).

RESULTS

Using the optimized PCR condition the amplification of the genomic DNA of all the 6 banana cultivars resulted in the generation of a single sharp fragment of around ITS2 fragment of 560 bp and rbcL fragment of 680 bp respectively.

Sequence Analysis for ITS2 sequences

The raw sequences obtained from the 6 samples on PCR amplification with the IT2 primers were obtained and analyzed with the corresponding chromatogram showed variable length in the

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order of Changthir (486 bp) > Lailaphu2 (461 bp) > Changvandawt (429 bp) > Lailaphu1 (394 bp) > Balhlasen (375 bp) > Banpawl (368 bp). The edited sequences obtained after analysis through Blastn and Blast2seq tools were in order of Lailaphu2 (312 bp) > Changthir (282 bp) > Lailaphu1 (241 bp) > Banpawl (215 bp) > Changvandawt (214 bp) > Balhlasen (203 bp). The homology search result of the edited sequences using MegaBlast program of the Blastn algorithm showed high value of identity (97-99%) with the Genbank database of ITS2 sequences from different banana species (Table 2). For the sequence analysis, the reference ITS2 sequences of *E. glaucum* (GI: 239584269) and *E. ventricosum* (GI: 332714902) retrieved from the genebank database were used as an outgroup. The average length of the sequences is 210.1 and the nucleotide composition were in the order of G (39.9) > C (30.4) > T (16.3) > A (13.4). *Musa laterita*1 and *Musa laterita*2 have the highest content of T nucleotide, *Musa ornata* cv Changvandawt have the highest content of C nucleotide, *Musa* AAB group cv.Balhlasen have the highest content of A nucleotide and *Musa laterita*1 have the highest content of G nucleotide. The multiple sequence alignment of all the ITS2 sequences including the samples studied and the outgroup revealed variability in sequence sites including nucleotide substitutions and indels occurred (Fig. 1). The ITS2 sequences were then again aligned using software MEGA 5.0 in order to study the variable nucleotide sites occurring in the ITS2 sequences among the banana samples studied. In this study, *Musa laterita*2 (Rhodochlamys) was used as a model. With the analysis of sequence variation in the commonly existing sequence, a total of 50 variable nucleotide sites were found where a sum total of 89 nucleotide changes was found to be occurring among the banana samples studied. In each site it was found that some of the nucleotides have been changed which could either deletion or insertion be as when compared to the model sequences i.e *Musa laterita*2. Among the banana samples studied,*Musa ornata* cv.Changvandawt (Eumusa) have the most variable nucleotide changes of 36, while *Musa* ABB group cv.Banpawl (Eumusa) have the least variable nucleotide changes of 10. The relative distribution between intra- and inter-species divergence of the ITS2 sequences in the banana samples was represented in a bar diagram (Fig. 2). The genetic distance within the range of 0.020 – 0.040 was observed to have the highest distribution with 2 instances at the intra-species level. At the inter-species level, the range of genetic distance between 0.160 - 0.180 was observed to have the highest occurrence with 6 cases. The phylogenetic relationships generated by the analysis of the ITS2 sequences using Maximum Likelihood (ML) method revealed the grouping of the samples into 2 clusters (Fig. 3). One cluster shows a bootstrap value of 99% existing between *E.glaucum* and *E.ventricosum* while the other cluster shows 4 sister clusters with a bootstrap value of 79% between Changtir (Eumusa) and Changvandawt (Rhodochlamy), 48% between *Musa Laterita1* and *Musa Laterita2* (Rhodochlamys) ,and 41% between Banpawl (Eumusa) and Balhlasen (Eumusa). The homology search result of the edited sequences using

Table 2: Result of the Blast search for similarity between the ITS2 sequences obtained from the banana samples studied and GenBank data carried out using the MegaBlast program of the Blastn algorithm.

Sl. No.	Sample	Gene Bank ID & Sequence name	Sequence identity	E value
1	*Musa* ABB group cv.Banpawl	GI:332715029 Internal transcribed Spacer	99%	3e-139
2	*Musa*AAB group cv.Balhlasen	GI:332715004 Internal transcribed Spacer 2	99%	1e-97
3	*Musa* BB group cv.Changthir	GI:343405463 Internal transcribed Spacer 2	99%	2e-141
4	*Musa ornata* cv. Changvandawt	GI:213136971 Internal transcribed Spacer 2	97%	7e-95
5	*Musa laterita* cv. Red (Lailaphu 1)	GI:213136957Intenal transcribed Spacer 2	98%	7e-122
6	*Musa laterita* cv. Green (Lailaphu2)	GI:213136957Intenal transcribed Spacer 2	99%	3e-166

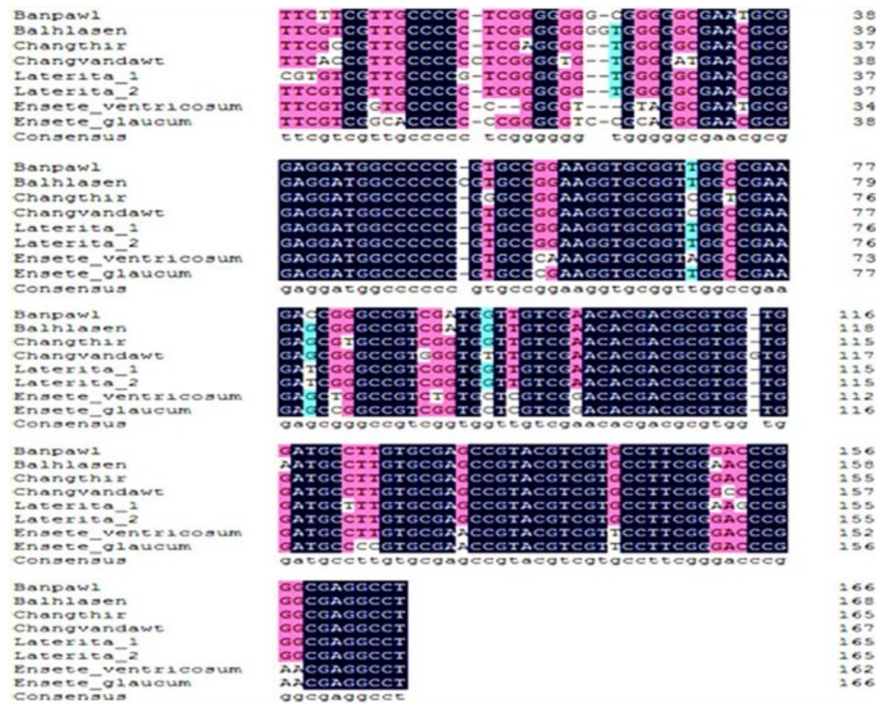

Figure 1. Multiple sequence alignment of the ITS2 sequences.

MegaBlast program of the Blastn algorithm showed high value of identity (95-99%) with the Genbank database of rbcL sequences from different banana species (Table 2). For the sequence analysis, the reference rbcL sequences of *E. glaucum* (GI: 227808823) and *E. ventricosum* (GI: 37722337) retrieved from the genbank database were used as an outgroup. Multiple sequence alignment of all the rbcL sequences including the samples studied and the outgroup revealed variability in sequence sites including nucleotide substitutions and indels occurred (Fig. 4).

Sequence Analysis for rbcL sequences

The sequence information of the unedited and edited sequences obtained from the 6 banana samples. The unedited sequences of the samples showed variable length in the order of Lailaphu2 (665bp) > Banpawl (660bp) > Changthir (614) > Balhlasen (597 bp) > Lailaphu1(575bp) > Changvandawt (413bp). The edited sequences after analysis through Blastn and Blast 2seq tools were in the order of Lailaphu2 (521bp) > Banpawl (520bp) > Balhlasen (513bp) > Changthir (510bp) > Lailaphu1(491bp) > Changvandawt (337bp). The homology search result of the edited sequences using MegaBlast program of the Blastn algorithm showed high value of identity (95-99%) with the Genbank database of rbcL sequences from different banana species (Table 3). Multiple sequence alignment of all the rbcL sequences including the samples studied and the outgroup revealed variability in sequence sites including nucleotide substitutions and indels occurred (Fig. 4). To study the variable nucleotide sites occurring in the rbcL sequences among the banana samples studied *Musa* ABB group cv. Banpawl (Eumusa) was used as a model for reference. With the analysis of sequence variation in the commonly existing sequence, a total of 39

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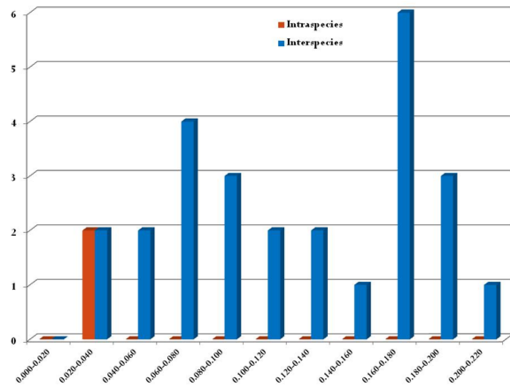

Figure 2: Relative distribution between intra- and inter-species divergence of the ITS2 sequences using 0.020 barcode distance units.

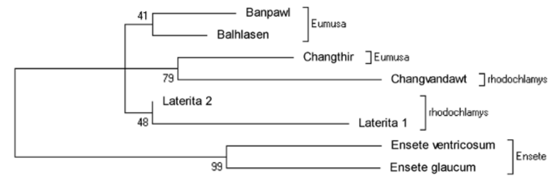

Figure 3: Phylogenetic tree generated by analysis of the ITS2 sequences using Maximum Likelihood method.

Table 3: Result of the blast search for similarity between the rbcL sequences obtained from the Banana samples studied and GenBank data carried out using the MegaBlast program of the Blastn algorithm

Sl. No	Species name	Gene Bank ID & Sequence name	Sequence identity	E value
1	Musa ABB group cv.Banpawl	GI:227808689Ribulose-1'5-bisphosphate carboxylase/oxygenase large subunit(rbcL)	99%	0.0
2	Musa AAB group cv.Balhlasen	GI:156598412Ribulose-1'5-bisphosphate carboxylase/oxygenase large subunit(rbcL)	99%	0.0
3	Musa BB group cv.Changthir	GI:227808689Ribulose-1'5-bisphosphate carboxylase/oxygenase large subunit(rbcL)	95%	0.0
4	*Musa ornata* cv. Changvandawt	GI:227808811Ribulose-1'5-bisphosphate carboxylase/oxygenase large subunit(rbcL)	99%	2e-174
5	*Musa laterita* cv. Red	GI:227808791Ribulose-1'5-bisphosphate carboxylase/oxygenase large subunit(rbcL)	99%	0.0
6	*Musa laterita* cv. Green	GI:227808791Ribulose-1'5-bisphosphate carboxylase/oxygenase large subunit(rbcL)	99%	0.0

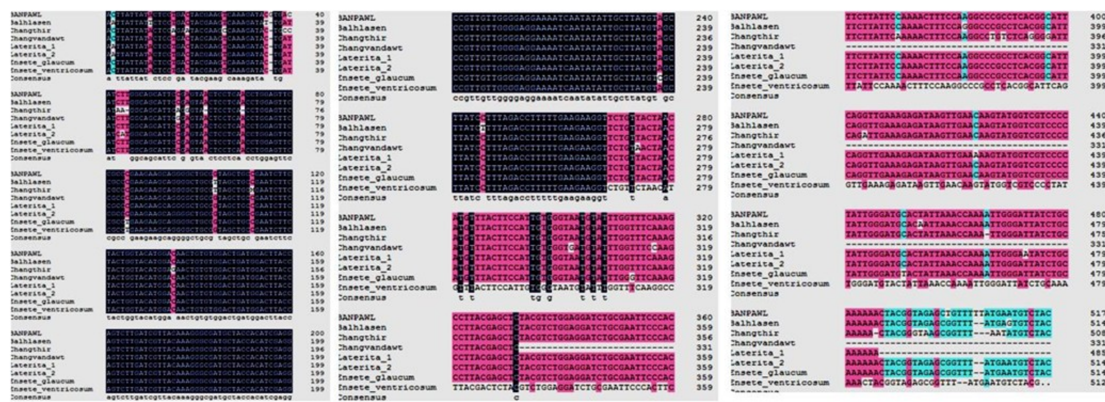

Figure 4: Multiple alignments of the rbcL sequences analyzed.

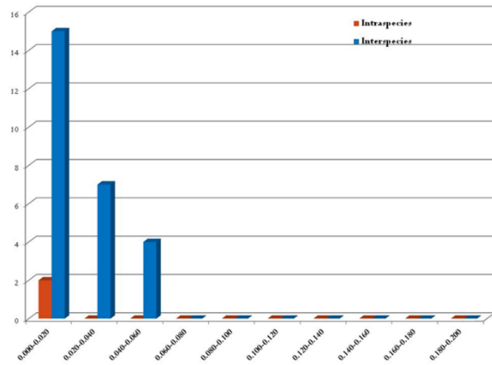

Figure 5: Relative distribution between intra- and inter species divergence of the rbcL sequences using 0.020 barcode distance units.

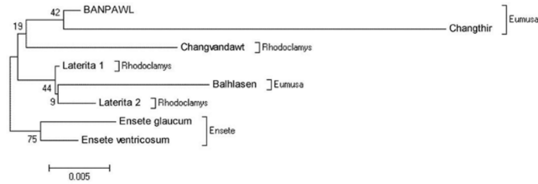

Figure 6: Phylogenetic relationship revealed by rbcL sequences of the banana cultivars.

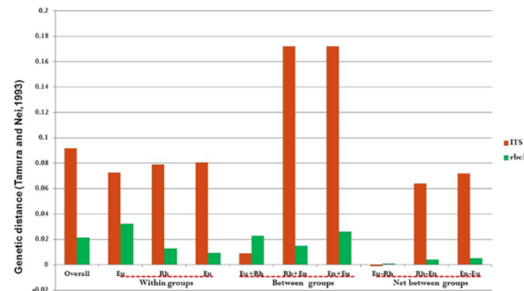

Figure 7: Genetic distance within and between groups of Musaceae as revealed by ITS2 and rbcL sequences in the samples studied.

Table 4: Analysis of intra and inter sections of Musaceae using ITS2 and rbcL gene sequences

Genetic Distance (Tamura and Nei, 1993)	**ITS2 (Mean ± S.E.)**	**rbcL (Mean ± S.E.)**
Overall genetic distance	0.0918 ± 0.0129	0.21 0.004
Distance within groups		
i)Eumusa	0.0725 ± 0.0235	0.032 ± 0.008
ii)Rhodoclamys	0.0788 ± 0.0231	0.012 ± 0.005
iii)Ensete	0.0807 ± 0.0308	0.009 ± 0.005
Distance between groups		
i)Eumusa + Rhodoclamys	0.069 ± 0.016	0.023 ± 0.006
ii)Rhodoclamys + Ensete	0.172 ± 0.041	0.015 ± 0.005
iii)Ensete + Eumusa	0.172 ± 0.042	0.026 ± 0.006
Net between groups		
i)Eumusa + Rhodoclamys	-0.001 ± 0.004	0.001 ± 0.002
ii)Rhodoclamys + Ensete	0.064 ± 0.018	0.004 ± 0.003
iii)Ensete + Eumusa	0.072 ± 0.019	0.005 ± 0.004

Table 5: Wilcoxon signed-rank test for inter-species divergence among loci.

W+	**W-**	**Relative ranks, n, P value**	**Result**
ITS2	rbcL	W+= 3, W- = 0, n = 3, p=0.109	ITS2 > rbcL

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variable nucleotide sites were found where a sum total of 96 nucleotide changes was found to be occurring among the banana samples studied. In each site it was found that some of the nucleotides have been changed which could either deleted or inserted as when compared to the model sequence. The pairwise genetic distance calculated using Tamura and Nei's (1993) model revealed the minimum distance between *M. laterita*1(Rhodochlamys) and *M. laterita*2 (Rhodochlamys) with a value of 0.003. The maximum distance value of 0.046 was observed in 3 cases between *Musa* AAB group cv. Balhlasen and *Musa* BB group cv.Changthir, *Musa* AAB cv. Balhlasen and *M. ornata* cv. Changvandawt, *Musa* BB group cv.Changthir and *E. ventricosum.* The relative distribution between intra- and inter-species divergence of the rbcL sequences in the banana samples was represented in a bar diagram (Fig. 5). The genetic distance within the range of 0.000 – 0.020 was observed to have the highest distribution with 2 instances at the intra-species level and 15 at the interspecies level. The phylogenetic relationships generated by the analysis of the rbcL sequences using Maximum Likelihood (ML) method revealed the same grouping of the sequences into 2 clusters with a low bootstrap value (Fig. 6) as compared to that generated by rbcL sequences. One cluster shows a bootstrap value of 75% existing between *E.glaucum* and *E.ventricosum* while the other cluster shows 4 sister clusters with a bootstrap value of 42% between Banpawl (Eumusa) and Changthir (Eumusa) , 9% between Balhlasen (Eumusa) and*M. laterita2* (Rhodochlamys) and Balhlasen (Eumusa), 44% between *M.laterita1* (Rhodochlamys) and sister clad(Balhlasen, laterita 2) and 19% between Changvandawt (Rhodochlamys) and another sister clad (Banpawl, Changthir).

Overall analysis of the ITS2 and rbcL sequences:

The overall analysis of the genetic distance observed at the intra- and inter-sectional levels of the Musaceae family using the ITS2 and rbcL genetic sequences obtained in the study are represented in (Table 4 and Fig. 7). The overall genetic distances across all the sections were observed to be 0.0918 with ITS2 and 0.021 with rbcL respectively. Comparison of the ITS2 and rbcL sequences in terms of its ability to generate inter-species divergence was calculated with Wilcoxon signed-rank test revealing the superiority of ITS2 over the rbcL (Table 5).

DISCUSSION

A favourable DNA barcoding must contain short sequence length to facilitate amplification and sequence, enough genetic divergence for identification and conserved regions for developing universal primers (Kress *et al*., 2005; Sass *et al*., 2007).

Due to the high primer universality and discriminatory power, the rRNA gene sequences and rbcL are found easy to access due to highly conserved flanking regions allowing for the use of universal primers (Chenuil, 2006). They are routinely used for phylogenetic studies (Schneider *et al*., 2004). In this study, the ITS2 and the partial *rbcL* region have been evaluated for their possible barcoding in the 6 members of Musaceae family with reference to 2 sequences of the *Ensete* genera as a possible outgroup. ITS2 was proposed by Kress *et al*. (2005) as a potential barcode for plants. The rbcL gene sequences have been recommended by CBOL as a candidate region for the barcoding of plants (CBOL, 2009). The rbcL marker proved to be easily in amplification and sequence with universal primers (Newmaster *et al*., 2006). A number of *rbc*L data are deposited in GenBank.

The analysis of the ITS2 and the rbcL sequences obtained from the PCR amplified fragments generated by the primer pair from the 6 banana samples using blast search revealed a very high identity percentage (97-99%) with the genbank database. For the proper evaluation of the relationship within the Musaceae family reference sequences of 2 species of the genus *En-*

sete were retrieved from the genbank database and used as outgroup. The *Ensete spp.* are far distinct from the species of *Eumusa* and *Rhodochlamys* section (Bekele and Shigeta, 2011). The overall analysis of the ITS2 and rbcL sequences revealed the insufficient variable, low interspecific variation and entirely no gap. Therefore the uses of these candidate genes (ITS2 and rbcL) were not successful in generating a substantial barcode for the Musaceae family. It is therefore suggested from this finding that the use of more markers and species for possible barcoding of the family.

CONCLUSION

The occurrence of various cultivars belonging to different genome groups in their wild and in cultivated sites further strengthened the observation that Mizoram as a part of the northeast Indian region constitutes the region where the natural hybridization of the banana and their wild relatives occurs. This work attempted using DNA barcodes (ITS2 of the nrDNA ITS region and rbcL of the chloroplast coding region) to evaluate phylogenetic relationship of the Musaceae family. Although rbcL have been recommended by CBOL as a candidate region in their last study (CBOL, 2009) it had a poor performance with insufficient variable, low interspecific variation and no gap. Thus, we suggested that this locus is not a god candidate for the Musaceae family. The ITS2 revealed relatively more variable nucleotide information than the rbcL DNA barcodes, although it cannot be considered as a DNA barcode for Musaceae family yet we came to conclusion that ITS2 is a much better locus than rbcL. The evolution of the members of the Musaceae family studied has been more clearly in combination with multilocus DNA barcodes than that the single-locus. Thus, this work not only provided more sequence sources of the Musaceae species, but helped further understand the phylogenetic relationship of this taxonomically complex family.

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Genetic diversity among edible banana varieties found in Mizoram, India

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ABSTRACT

Banana is considered as an important crop plant due to its high economic value as good dietary source. The distribution and diversity among the cultivated and wild varieties are largely influenced by environmental factors. Therefore, the present study is undertaken to analyze the genetic diversity among seven different cultivated non-seeded banana varieties that are grown in the field gene bank of Department of Biotechnology, Mizoram University, Aizawl, Mizoram. RAPD technique was employed for studying the genetic variation among edible banana varieties such as Vaibalhla (VB), Banria (BR), Lawngbalhla (LB), Banthur (BT), Kawlbalhla (KB), Banpawl (BP), and Balhlasen (BS). Six RAPD primers used for the present studies led to the amplification of DNA polymorphism. Out of six primers used, four primers namely UBC 416,418, 419 and 420 generates 100% polymorphism in all the samples. The dendrogram analysis reveals two major groups and shows highest similarity between the samples Banria (BR) and Banpawl (BP). Using RAPD technique it was shown that significant amount of genetic diversity existed in the samples studied, thereby providing clear genetic information for future breeding and conservation strategies.

Key words: Banana, RAPD, Genetic diversity, Dendrogram, Mizoram

INTRODUCTION

Banana is regarded as one of the favorite fruit crops of the world and is widely distributed throughout the tropics growing across the world with more than 120 countries. Its total production from nearly 10 million hectres reached approximately 106 million tonnes per year (Molina and Kudagamage, 2002). It is the fourth most important crop after rice, wheat, and maize for millions of people (Uma and Sathiamoorthy, 2002). It is strongly believed that banana have originated from Southeast Asia (Sathiamoorthy *et al*., 2001). Clarke (2001) stated that most banana cultivars reproduce asexually, and some varieties long been in cultivation for upto 8000 years. Nearly half of the world banana production is cultivated in Asia, while almost 75% of the world's plantains are cultivated in Africa. Edible banana along with ornamental ones and plantains belong to the family of *Musaceae*, which consists of two genera: *Musa* and *Ensete*. Genus *Ensete* has 9 species while *Musa* genus has four sections namely - Eumusa, Rhodochlamys, Australimusa and Callimusa (Simmonds and Shepherd, 1955). Eumusa constitutes the sources of edible banana chiefly belonging to *Musa acuminata* Colla and *Musa balbisiana* Colla. Edible banana originated from the inter- and intra-specific hybridization of wild species *M. acuminate* and *M. balbisiana* (Ashalatha *et al*., 2005). Depending on the contribution of *M. acuminata* (AA) and *M. balbisi-*

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ana (BB), the cultivars have been classified into genomic groups (AA, AAA, AAB, ABB, BB, AB, BBB, AAAA and ABBB).

India is the leading country in global banana production with an annual production of 13.5mt from an area of 4.0 lakh ha and is further focusing to achieve 25 mt by the end of 2020 in production level (Sathiamoorthy *et al*., 2001). India has a rich genetic diversity of banana with more than 90 distinct clones. The northeastern region of India have been considered as the richest sources of natural banana diversity where *M. balbisiana* from Indian subcontinent meet *M. acuminata* from South East Asia (Molina and Kudagamage, 2002). Besides the cultivated species and their cultivars, majority of the species of *Musa* were found in wild condition and they are widely distributed in the northeast states. Mizoram is located in the northeastern corner of India, enjoying the tropical, sub-tropical and temperate region in the state. Wild and edible banana varieties are abundantly distributed throughout Mizoram. Therefore, the level of genetic diversity of banana in the state as a result of several potential nature such as hybridization, mutation, and polyploidy is an area for scientific studies.

There have been several reports of earlier works on the studies of genetic makeup of bananas by using molecular marker technique such as randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and microsatellites (Onguso *et al*., 2004; Wong *et al*., 2002). Uma *et al*., (2005) have also reported the evidence of genetic variation successfully in Indian wild *Musa balbisiana* (BB) population by using RAPD markers. The use of RAPDs in molecular biology have the advantage that the material is processed by an efficient and inexpensive technique without requiring prior knowledge of the genome (Bhat *et al*., 1995). RAPD assay has the advantage of being easy to use, requiring very small amount of genomic DNA without the need for blotting and radioactive detection and are moderately reproducible (Cipriani *et al*., 1996). Thus, the present study was undertaken to analyze the genetic variation of edible cultivated banana varieties maintained in the field gene bank of the Department of Biotechnology, Mizoram University using RAPD markers.

MATERIALS AND METHODS

Plant Material

For the present study, seven cultivated non-seeded banana samples were selected from the field gene bank in the Department of Biotechnology, Mizoram University, Aizawl. The samples are Vaibalhla (VB), Banria (BR), Lawngbalhla (LB), Banthur (BT), Kawlbalhla (KB), Banpawl (BP), and Balhlasen (BS).

DNA extraction

The young leaves were collected from the banana plants and used for DNA extraction. Leaves weighing approximately about 200 mg were used in the DNA extraction using the protocols of Thangjam *et al*. (2003). The leaves were excised and washed with double distilled water and then put into the micro-centrifuged tube (2 ml) containing 400µl of extraction buffer. The tube containing the leaf samples was incubated at 37 °C for at least 90 min in water bath and homogenized with a glass rod inside the tube. 400µl of 2% CTAB solution was added and incubated at 65 °C for two hours. It was cooled to room temperature, then equal amount of phenol: chloroform: isoamyl alcohol (25:24:1) was added and centrifuged at 10000 rpm at 4 °C for 10 min. The upper aqueous layer was carefully transferred to a new tube and the step was repeated 3 times to remove the cloudiness of upper layer. Then 2/3rd volume of icecold isopropanol was added, mix gently by repeated inversions and incubate at room temperature for 30 min to precipitate DNA. It was again centrifuge at 10000 rpm for 5 min at 4 °C and the supernatant was discarded. The pellet was washed with 70% alcohol and pellet was air dried. The pellet was resuspended in 300 µl of TE and air dried at 37 °C

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Table 1: Details of amplification bands obtained with different UBC primers in the banana samples

Primer	Sequence (5´ - 3´)	Number of amplified bands	No. polymorphic bands	Percentage of polymorphism
UBC-416	GTGTTTCCGC	13	13	100
UBC-418	GAGGAAGCTA	14	14	100
UBC-419	TACGTGCCCG	11	11	100
UBC-420	GCAGGGTTCT	14	14	100
UBC-421	ACGGCCCACC	24	20	83.3
UBC-450	CGGAGAGCCA	16	14	87.5
	Total	92	86	95.14

for approximately 1hr. 1-5µl of RNase (100 mg/ ml) was added to remove RNA and incubate at 37 °C for 30 min.

DNA quantification

The quantity of the extracted DNA was estimated using Biophotometer plus (Eppendorf, Germany) by taking their absorbance at 260 nm and 280nm. 2µl of extracted DNA, diluted in 48µl of TE buffer in a sterile cuvette, was used to obtain the concentration of DNA in all the samples. The concentration is in terms of ng/µl. The ratio of absorbance at 260 nm to the absorbance at 280 nm was taken as reference for the purity of the extracted DNA. A ratio between 1.6 and 1.9 was regarded as a pure sample, free from unwanted RNA/ protein contamination. For each sample, a 100ng/µl stock was prepared from the original mother stocks for further analytical procedures.

PCR amplification using random primers

The DNA was diluted to 10 ng/µl from the stock of 100 ng/µl. The PCR reaction mixture was assembled in a 25 µl volume containing 5µl each of extracted DNA (10 ng/µl) samples, 13.59µl of sterile millique water, 2.5µl 1X Taq buffer,1.5µl dNTPs (0.5mM), 2µl $MgCl_2$ (2mM), 0.33µl UBC (University of British Columbia, Cananda) RAPD primer (50 pmol), 0.33µl Taq polymerase (1U) was prepared. The thermocycler (Applied Biosystems, USA) was programmed at 1 cycle of 94 ^{0}C for 1 min and then 35 cycles of 94 ^{0}C for 1min 36 ^{0}C for 50 sec and 72 ^{0}C for 2 min. The last extension cycle was programmed at 72 ^{0}C for 4 min. The amplified products were electrophoresed on a 1.8% ethidium bromide stained agarose gel in TBE buffer by loading 15µl of the products with 3µl of loading dye. The gel was viewed and then photographed using a gel documentation system (UVP Ltd., UK).

Analysis of the RAPD profiles

The amplified products were scored for the presence or absence of DNA fragments. When a fragment was present at a specific fragment size, it was assigned a '1'. If the fragment was absent at that fragment size, then, it was score as a '0'. Data generated from the RAPD profiles were analyzed using Nei and Li's coefficient. A dendrogram was constructed on the basis of the similarity matrix data by UPGMA (unweighted pair group method with arithmetic average) method. All the analysis was conducted using the software NTSYS-pc version 2.2 (Numerical Taxonomy and Multivariate Analysis for Personal Computer Version 2.2) (Rohlf, 2000).

RESULTS AND DISCUSSION

The present study was carried out in the cultivated non-seeded banana samples of Mizoram maintained in the field gene bank in the Department of Biotechnology, Mizoram University, Aizawl, to analyze the genetic diversity. DNA was isolated following the protocol provided by

Table 2: Dissimilarity matrix obtained from RAPD profile in the banana samples studied (VB= Vanbawl, BT= Banthur, BS= Balhlasen, BR= Banria, BP=Banpawl, KB= Kawlbahlha, LB= Longbalhla)

Sample	VB	BR	LB	BT	KB	BP	BS
VB	0.00						
BR	0.646	0.000					
LB	0.861	0.442	0.000				
BT	0.484	0.543	0.825	0.000			
KB	0.499	0.511	0.621	0.719	0.000		
BP	0.603	0.266	0.520	0.769	0.400	0.000	
BS	0.545	0.546	0.644	0.503	0.557	0.523	0.000

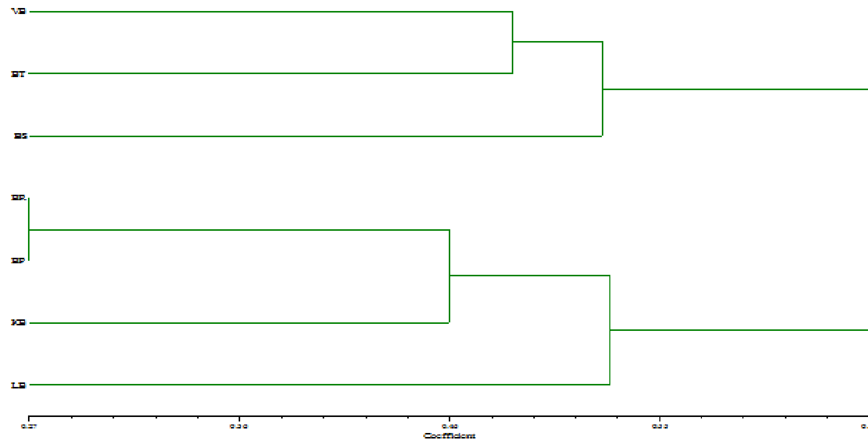

Figure 1: Dendrogram resulted from the analysis from RAPD analysis (VB= Vanbawl, BT= Banthur, BS= Balhlasen, BR= Banria, BP=Banpawl, KB= Kawlbahlha, LB= Longbalhla)

Thangjam *et al*., (2003) with minor modifications. RAPD markers were used for analyzing the genetic variations.

The PCR amplification products using 6 RAPD primers in the seven banana samples was resolved on 1.8% agarose gel. The details of the RAPD profile generated in the banana samples studied are given in Table 1. A total of 92 fragments were scored out of which 86 were. polymorphic (93.47%). This amplification result indicates that the randomly arranged nucleotides sequences of nucleotides (primers) have their complementary pairing with the isolated DNA from the samples. The patterns of their dissimilarity in amplifications signify that they are genetically asymmetric. Selective amplification products in PCR condition by using random primers established that the tested DNA samples were diversified from one another. Different samples produce different numbers of bands with a particular primer. 13 amplified bands obtained from primer UBC-416 generated 100% polymorphism. It is found that primer UBC-418 produced 14 polymorphic bands generating 100% polymorphism. While UBC-419 primer exhibited 11 amplified products and UBC-primer 420 produced 14 amplified products. The percentage polymorphism using the primers UBC-419 and -420 were found to be 100%. UBC-421 primer generated 24 amplified bands and 20 polymorphic bands with 83.3% polymorphism. UBC-450 primer produced 16 amplified bands and 14 po-

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lymorphic band with 87.5% polymorphism.

The Nei and Li's dissimilarity coefficient between the samples are given in Table 2. The highest similarity was obtained between the sample BR and BP scoring the dissimilarity index of 0.26% where as the lowest similarity is found between the sample of LB and VB scoring the dissimilarity index of 0.86%. The scores obtained from the RAPD profiles were used to construct a dendrogram using unweighted paired group method of cluster analysis using arithmetic averages (UPGMA). The resulted dendrogram revealed the clustering of the 7 samples into two distinct groups (Figure 1). The first group consists of BR, BP, KB and LB banana varieties while the second group consists of VB, BT and BS varieties.

The present study was primarily focus on the study of genetic variation among the cultivated edible banana varieties from locally available banana in Mizoram. Using RAPD technique it was shown that significant amount of genetic diversity existed in the samples studied, thereby providing clear genetic information for future breeding and conservation strategies.

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